Screening Environmental Pseudomonads for Characteristics Suitable for a Bio-engineered Oil Remediation Agent

By

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B Eng., MSc

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DEDICATION

This dissertation work is dedicated to my loving and caring children for given me joy when am bored writing and resuscitating me back on track;

Ummu-salma (Mami), Umar-Farouk (Amir), Halimatu-sadiya, Abubakar-sadiq (Bappa/Sarki), Sumayya, Ibrahim (Mallam), Fatimatu-zahra'u, and Muhammad

AUTHOR'S DECLARATION

I, **Ibrahim Umar Mohammed** hereby wilfully declare that the following thesis is based on the results of research wholly conducted by myself, and that it is of my own composition. This thesis has not, in whole or in part, been previously presented for a higher degree or qualification. All works used in the course of this work are evidently referenced as appropriate.

……...

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CERTIFICATION

I hereby certify that Ibrahim Umar Mohammed, a PhD candidate has undertaken all the work described herein and is based on the original work done at the Abertay University, Dundee in partial fulfilment for the requirements for the award of Doctor of Philosophy in Environmental Bioremediation. This has not previously formed the basis for the award of any degree, diploma, assistanceship, fellowship or any similar title and represents an independent work on the part of the candidate.

Signature of the Principal Supervisor……...

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LIST OF ABBREVIATIONS AND ACRONYMS

A – Absorbance

AD – Anderson-darling statistics

AD* – Adjustted Anderson-darling statistics

A–L – Air-liquid interface

ANOVA – Analysis of variance

A_i – Aqueous index

E_i – Emulsion index

EPS – Extracellular polymeric substances

HCA – Hierarchical cluster analysis

IDI – Individual distribution identification

LSTRA – Low surface tenstion reducing activity

OD – Optical density

O_i – Oil index

P – Predicted value

r – Correlation coefficient

SE– Standard error

SUDS – Sustainable urban drainage systems

TK-HSD – Tukey Kramer Honest Significant Difference

ULO – Used lubricating oil

γ_{Min} – Minimum surface tension

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ABSTRACT

Biosurfactants are surface active chemicals expressed by a range of organisms that reduce liquid (aqueous) surface tensions (γ) of aqueous and aqueous-hydrocarbon (oil) mixtures. They are widely used in biotechnology, including agriculture, cosmetics, food, pharmacology and bioremediation, and many new biosurfactants are identified through surveys of bacteria recovered from a variety of environments. In this work, environmental *Pseudomonas* spp. were screened for biosurfactant production and behaviours determined in order to investigate the limits of biosurfactant activity and potential structural diversity within a phylogenetically related group of bacteria.

A total of 355 pseudomonads and *Pseudomonas*-like isolates were isolated from activated sludge wastewater and potentially petroleum-contaminated soils from road side drainage (SUDS) site. These were phenotypically characterized using a number of growth and behaviour assays, including air-liquid interface biofilm formation in static microcosms and in a column bead system, and shown to be a diverse collection of isolates with a minimal level of biological replication (i.e. little evidence of identical strains recovered more than once in or between samples). Of these, 57 isolates were found to express biosurfactants *in vitro* by drop-collapse assay and confirmed by quantitative tensiometry. The surface tension of cell-free culture supernatants produced by these isolates was between 24.5 – 49.1 mN m^{-1}, with a minimum theoretical surface tension ($\gamma_{Min)}$ of 24.2 mN m^{-1}. This is in agreement with earlier predictions, suggesting a fundamental limit to the ability of bacterial biosurfactants to reduce surface tensions in aqueous systems. This finding suggests that further effort to isolate stronger active surfactants are likely to be wasted, and poses the interesting question of what biological or physical factors limit the production of stronger biosurfactants by bacteria.

Differences in biosurfactant behaviour determined by foaming, emulsion and oil-displacement assays were also observed amongst select isolates producing surface tensions of 25 – 27 mN m^{-1}, suggesting structural diversity in the biosurfactants produced. These findings provide a system for selecting biosurfactants for further chemical-structural analyses and future testing for various biotechnology applications where low surface activity, but varied behaviour is required.

Chapter 1

Introduction

1.1 An introduction to this thesis

Bacteria are capable of producing a range of substances and be used for a number of processes in a variety of biotechnology applications (reviewed by Erickson & Winter, 2012; Gurung *et al.,* 2013). The use of bacteria has also led to the production of various enzymes with increased activities and flexibility that can also produce chemicals of value (Gurung *et al.,* 2013).

The research work presented here is focused on bacterial biosurfactants which are used in a range of biotechnological applications including bioremediation. Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic substances, water bioavailability and change the properties of the bacterial cell surfaces. The surface activity of biosurfactants make them excellent emulsifiers, foaming and dispersing agents, which are useful in a range of biotechnological applications in agriculture, cosmetics, pharmaceutic, food technologies, and bioremediation of hydrocarbon contaminated sites (Banat *et al.,* 2000).

In bioremediation biosurfactants have advantages over chemically synthesized (inorganic) surface active compounds, as they are more biodegradable and often less toxic than the chemically synthesized versions (Banat, 1995).

Biosurfactants with a wide range of liquid surface reducing activities, foaming and emulsification properties, can be used for the cleaning of hydrocarbon spills (e.g. oil, diesel, petrol) on surface and ground water, contaminated soils, beaches and sediments, and can also be used to enhance oil recovery from underground reservoirs (Pacwa-Plociniczal *et al.,* 2011).

Waste oils are generated in service stations, garages, new car dealer showrooms, other retail establishments and automotive fleet service areas (Owiti *et al.,* 2015). The waste oils comprise mainly of Crankcase oil, waste transmission fluids, gear lubricants, hydraulic oils, and minor amounts of solvents used in the service areas. Such Waste Oils are flammable and contain toxic ingredients (Owiti & Ndiritu, 2015; Speight & Exall, 2014). In highly industrialized areas, substantial amount of Waste Oils is generated from trucking, transportation and Construction Company fleets of service vehicles (Leong & Laortanakul, 2003). In the organized sectors like mass public transportation establishments, public works departments of various states, state transport corporations, defence services, airports, ports, mining and large industries, huge quantities of Waste Oils are generated (Owiti *et al.,* 2015).

This work is focused on bacterial biosurfactants produced by environmental pseudomonads, which are known to express a range of different surfactant types. This thesis builds upon earlier work which predicted that pseudomonad biosurfactant activity (i.e. strength) is limited to a minimum of ~ 24 mNm^{-1}, though no understanding is yet available to suggest why these bacteria cannot produce more active compounds resulting in lower surface tension. The diversity of biosurfactant behaviour would also be investigated as another area

of interest, using assays such as oil displacement, foaming and emulsion tests as a means of identifying novel biosurfactants for potential biotechnology applications in future work.

In this thesis introduction, bioremediation was chosen as a means of introducing biosurfactants, as this was the initial interest of this work at the start of the studentship. Since then, the project has become more focused in the prediction and behavioural aspects of biosurfactants, and how novel compounds could be identified for use in biotechnology.

1.2 The origins of environmental pollution

The introduction of substances into the environment with negative adverse effects causing damage to biotic and abiotic components of the environment is referred to as pollution. Although some pollutant may be natural, for example, volcanic gases and ash, having a prolonged and sometimes permanent impact on the planet earth, most pollutants are the result of human activity (Berger, 1997; Marcano *et al.,* 2003). The 18th century world population explosion that witnessed a hasty increase, from 1 billion to 7 billion between 1900 and 2000, has also contributed to the increase in soil and water pollution (Pimentel *et al.,* 2007). The industrial revolution and growing technology has brought about increase in the use of chemical substances such as petroleum oil and hydrocarbons which also increases the burden of environmental pollution (Boyd, 2015). It has been estimated that the world waters receive about 1.7 to 8.8 million metric tons of oil per year of which more than 90% is the result of

accidental discharge due to inefficiency and intentional indiscriminate disposal of waste (Zhu *et al.,* 2001; Ketkar, 2002; Agramuthu & Dadrasnia, 2013).

As the human population increases on a daily basis, there is a direct consequence of increased pressure on air, water and land resources (reviewed by Falkenmark & Widstrand, 1992). To satisfy the unlimited demands of the people, the hasty expansion of industries, food, health care, vehicles, etc. became a necessity. These developments have resulted in greater challenge to the quality of life and are not favourable to the environment where we live, if care is not taken to put in place good management systems (reviewed by Tolba *et al.,* 1992). However, when pollution occurs the immediate response on how to clean up the mess, since the quality of life is intricately linked to the overall quality of the environment (reviewed by Marans, 2015). Globally, attention has now been focused on finding sustainable ways of preserving the environment (reviewed by Turner *et al.,* 2007). These efforts could be possible by exploiting the use of biotechnology.

1.3 Bioremediation of hydrocarbon contaminated soils and other environments

Bioremediation is a biological process which involves the use of microorganisms to degrade, transform, or immobilize contaminants from soil and water environment into less harmful forms (see reviews by Khan, 2014; Divya *et al.,* 2015). Microorganisms (i.e. bacteria, yeasts and fungi) are involved in the breakdown of organic compounds naturally, but the process is often slow (reviewed by King *et al.,* 2015). In contrast, there are a number of non-biological

methods that could be applied to remove contaminants from the environment, including soil vapour extraction, air-sparging (this might also promote bacterial metabolism and the degradation of contaminants), thermal desorption, chemical oxidation and reduction, steam stripping, supercritical fluid extraction, ultraviolet (UV) radiation, electrokinetic remediation and photocatalytic degradation (see reviews by Fox, 1996; Gomes *et al.,* 2013).

Most of the microorganisms used in biodegradation of contaminated environments are members of the following genera: *Acinetobacter, Actinobacter, Alcaligenes, Arthrobacter, Bacillus, Berijerinckia, Flavobacterium, Methylosinus, Mycobacterium, Mycococcus, Nitrosomonas, Nocardia, Penicillium, Phanerochaete, Pseudomonas, Rhizoctonia, Serratia, Trametes* and *Xanthobacter* (see reviews by Korda *et al.,* 1997; Kulshreshtha *et al.,* 2014; Singh *et al.,* 2014).

Bioremediation methods are classified into *in situ* or *ex situ* techniques. The *in situ* technique involves the treatment of the contaminated material at that particular place or site (i.e. without removal), while *ex situ* treatment of the contaminated material occurs at a different site after physical removal (reviewed by Boopathy, 2000). In many *in situ* and *ex situ* techniques a ‘’bioremediation agent’’, such as a naturally-isolated or genetically modified bacteria having good contaminant degrading properties can be added to enhance natural degradative processes. It should be noted that changes to the environment in which the bioremediation active bacteria are present will effect growth and degradative characteristics. Changes in temperature, pH, water availability, the addition of nutrients and co-factors etc, can all have to be manipulated to improve

performance. Some of the examples of *in situ* and *ex situ* bioremediation of environments such as naturally containing pollutants of hydrophobic origin (including used lubricating oil which is often used as a model contaminant) are given below.

Biostimulation involves the adding of nutrients or other materials to stimulate the natural attenuation processes in which the microbial population take advantage of the added nutrients etc. to remediate contaminated soils by directly or indirectly degrading the pollutants (reviewed by Tyagi *et al.,* 2011). While bioaugmentation involves introducing microorganisms isolated elsewhere capable of cleansing a particular contaminant into a contaminated site. In some cases genetically modified microorganisms are used in bioaugmentation (reviewed by Kulshreshtha *et al.,* 2014). This is realized by creating an artificial combination of genes that do not occur together in nature, using a number of methods such as engineering with single genes or operons, pathway construction and modifications of the sequences of existing genes (reviewed by Wasilkowski *et al.,* 2012).

Phytoremediation is an *in situ* technique that makes use plants to treat soils and ground water contamination. This technology is applied in areas where remediation options are not cost effective and with low levels of contaminations. Deep rooted plants, grasses, legumes and some aquatic plants are normally used in phytoremediation fields. It has been used to remove organic compounds such as total petroleum hydrocarbons (TPH), poly aromatic hydrocarbons (PAH) and 2,4,6-tetrinitrotoluene (TNT) through translocation in plants by storing the chemicals and its fragments into the plants and

subsequent convertion to carbon dioxide and water (see reviews by Schnoor *et al.,* 1995; Susarla *et al.,* 2002). The plants remove pollutants from soils and ground water, and retain, metabolize or volatilize them. The root system helps a variety of microorganisms in the subsurface by providing organic carbon to enhance metabolism in the rhizosphere (see reviews by Schnoor, 1995; Ghosh & Singh, 2005). Other *in situ* bioremediation techniques include bioventing and biosparging.

Land farming involves the excavation of the contaminated material on to the soil surface in which the soil surface is constantly tilled and turned over repeatedly thereby exposing a new layer of soil to air. The constant agitation of the soil by tilling and mixing aerate the soil and pave way for microbial growth and activity in which the contaminants are degraded (Reviewed by Gan *et al.*, 2009). The moisture content of the soil is controlled by irrigation and spraying and pH by adding crushed limestone, when the soil is acidic due to breakdown of organic matter (Khan, 2014). This is normally used to treat soils contaminated by petroleum derived products (see reviews by Ausma *et al.,* 2003; Khan, 2014). The technology is simple, inexpensive, requires large space and in most cases contaminants made up of lighter hydrocarbons, including gasoline and other volatile organic contaminants, e.g. chloroform, carbon tetrachloride may vaporize rather than being treated by bioremediation (see reviews by Khan *et al.,* 2004; Maila & Cloete, 2004).

Biopiles are another form of treatment applied to contaminated soils, in which the contaminated soil is excavated and piled in heaps usually 2 to 3m high and confined to a lined area to prevent seepage of the contaminants. Biopiles are

aerated by forcing air to circulate by injection through perforated piping fitted throughout the pile or the piles placed over an aeration system (Naseri *et al.,* 2014). The movement of air within the piles do not only make oxygen available to the microorganisms present in the soil but also remove some contaminants from the soil. Moisture and nutrient levels are conserved, while the temperature of the system can be controlled (see reviews by Beames *et al.,* 2014; Kulshreshtha *et al.,* 2014). In this system, bioremediation agents (bacteria) can also be introduced through pipes delivering nutrients to enhance the degradation process.

Composting is a process in which the contaminated soil is excavated and then mixed with bulking agents which is then referred to as the compost material. They include hay, straws and corn cobs. This helps the microorganisms involved in the clean-up process to maintain optimum levels of air and water. The organic materials are selected based on their ability to provide enough porosity, and carbon and nitrogen balance to aid breakdown of contaminants. This process is normally accompanied by increased in temperature due to microbial activity that triggers biochemical reaction that favours degradation through microbial aerobic decomposition of the organic materials to humus like end product (see reviews by Tuomela *et al.,* 2000; Kulshreshtha *et al.,* 2014). Other *ex situ* bioremediation techniques includes bioreactors and biofilters.

Bacteria with the ability to utilize and degrade different hydrocarbons and with great potential to withstand harsh environments are the *Pseudomonas* and are known to express biosurfactants (Poli & Nicolaus 2010; Bustamante *et al.,* 2012). Previous studies have shown that biosurfactants solubilize and helps in

the removal or dispersion of hydrocarbons. It is also known to have effect on the cell surface hydrophobicity of bacteria by facilitating the attraction between microbial cells and hydrocarbons.

1.4 The involvement of bacteria in bioremediation

1.4.1 A brief introduction to bacteria for the non-microbiologist

Bacteria, are group of microscopic unicellular organisms that live in large numbers and are found almost everywhere on Earth (see reviews by May, 1988; Fenchel & Finlay, 2004). Bacteria are prokaryotes, defined by the lack of organised nucleus and other internal structures, while the eukaryotes are organisms that possess a membrane bound nucleus that holds genetic materials as well as membrane bound organelles. Bacteria have exceptionally diverse metabolic capabilities and can utilize organic and some inorganic compounds as a source of food or energy (reviewed by Ghafari *et al.,* 2008). Some bacteria are recognised as disease causing organisms in humans and other animals, or plants, but in general most of them are harmless and are beneficial environmental mediators whose metabolic activities sustain higher forms of life (reviewed by Rao *et al.,* 2010).

Some bacteria are found living in symbiotic relationships with plants and invertebrates, i.e. bacteria living in the hindgut of termites and *Rhizobium* bacteria in the roots of leguminous plants, where they serve their host by mediating nitrogen fixation and cellulose degradation (Brauer *et al*., 2015). Without bacteria, soils would not be fertile and the decay of organic material

would proceed at a very slow rate (Abed *et al.,* 2015). Bacteria are widely used in the preparation of food and in the manufacture of antibiotics and chemicals (reviewed by Sarter *et al*., 2015). Bacteria are widely known to secrete products (including lipases and surfactants) that help mobilise and degrade hydrocarbons. A schematic diagram of how surfactants influence some transport processes during bioremediation of organic contaminated soils is shown in Figure 1.1.

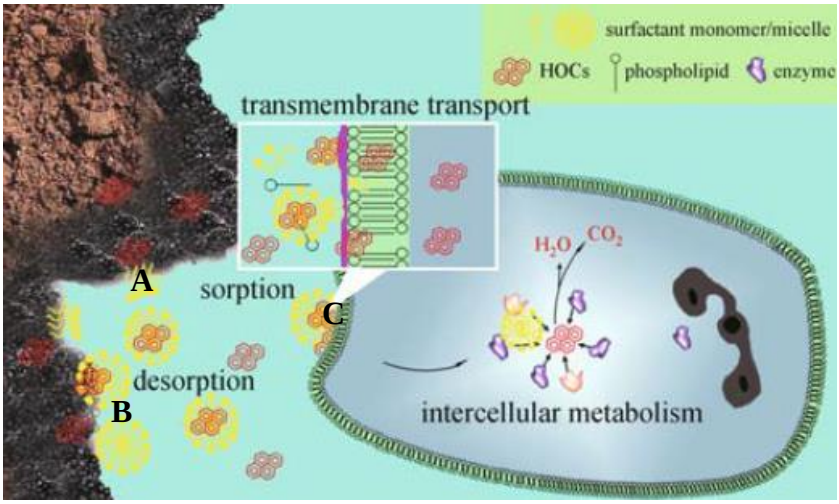


Figure 1.1 Shown are several Interface processes involved in surfactant mediated bioremediation by bacteria of hydrophobic organic contaminants (HOCs). In this figure the large oval represents a bacterial cell close to a solid soil particle which has absorbed onto its surface a hydrophobic organic contaminant such as oil. **(A)** Surfactant monomers expressed by the cell assemble at the particle-water and particle-HOC interfaces and allows desorption from the solid surface. (**B)** The desorbed oil droplets surrounded by surfactant molecules are then transported across the cell membrane **(C)** where the oil is then used for metabolism releasing carbon dioxide and water (Adapted from Zhang & Zhu, 2014).

1.4.2 Bacterial biofilms

Biofilms can be described as a community of bacteria and other single celled organisms, that are living attached to a surface, encircled in a matrix involving a combination of polymeric compounds, predominantly polysaccharides and commonly referred to as extracellular polymeric substances (EPS) and may involve single species or a corporation of species (reviewed by Lin *et al*., 2014).

Many studies on biofilm formation in the past have focused on single species structures (Davey & O'toole, 2000). However, in natural environments, biofilms are aggregates of more than one species and a lot of work has been done on these mixed species structures (Rao *et al*., 2005). Greater chances of survival is expected in biofilm compared to planktonic microorganisms because they are encased in a protective matrix that allows them to benefit more from the physiological processes taking place within structure (Singh *et al*., 2006). Biofilm involvement in bioremediation of petroleum hydrocarbon compounds has become an area of interests to researchers in recent times. They grow in close association with oil aggregates in sediments, sand, soil or on water (Macedo *et al.,* 2005; Yergeau *et al.,* 2013). *Pseudomonas* species are best known to produce surfactants, the enzyme lipase and form biofilms and as a result may be particularly useful as bioremediation agents. A summary of some of the main characteristics of biofilms are shown in Table 1.1.

Biofilms develop on a range of surfaces (i.e air-solid surface and air-liquid interfaces). Those grown at the surface of static liquids (i.e air-liquid interface biofilms) can be classified according to visual characteristics and response to physical disturbance (Figure 1.2).

Table 1.1 Major characteristics of biofilms with importance for bioremediation

Property	Characteristic	Reference
Tolerance towards environmental stressors (e.g., toxic chemicals, pH change, predation, and dehydration)	Extensive genetic diversification of biofilm bacteria	Boles *et al.* (2004), Vu *et al.* (2009), Boon *et al.* (2003), Accinelli *et al.* (2012), & Hall-Stoodley *et al.* (2004).
Communication (quorum sensing)	Critical cell density for biofilm formation	Hentzer *et al.* (2003) and Sarkar & Chakraborty (2008)
Exchange of genetic material	Horizontal transfer of genetic material between species, DNA sharing	Beaudoin *et al.* (1998), Ghigo (2001), & Wolcott *et al.* (2013).
Metabolic diversity and symbiosis	Utilizing waste products and/or accumulated products from the environment or other micro-organisms	Von Canstein *et al.* (2002), Boles *et al*. (2004), Picioreanu *et al.* (2000), & Chen *et al.* (2013).
Redox and electron acceptor diversity	Different metabolic functions with respect to electron-acceptor reduction	Falkentoft *et al.* (2002), Zhang *et al.* (2010), and Ontiveros-Valencia *et al.* (2012).
Varying growth rates in the biofilm	Inducing biofilm persistence due to different metabolic states in the biofilm	Wentland *et al.* (1996), Davies *et al.* (1998), Costerton (1999), and Woo *et al.* (2012).
Porous physical structure with water channels	Allow for transport of nutrients, electron acceptors, and waste products	Latch *et al.* (2003), Hall-Stoodley *et al.* (2004), Wijeyekoon *et al.* (2004), Seo *et al.* (2009), Wang *et al.* (2011), and Xiao *et al.* (2012).
Surfactants	Aid in solubilizing hydrophobic or recalcitrant substrates	Rodriguez and Bishop (2008) and Seo *et al.* (2009).
Microcolony and gradient formation	Redox potential and nutrient cycling due to aerobic and anaerobic process	Galiana e*t al.* (2008) and Verhagen *et al.* (2011).

Source: Edwards & Kjellerup, (2013)

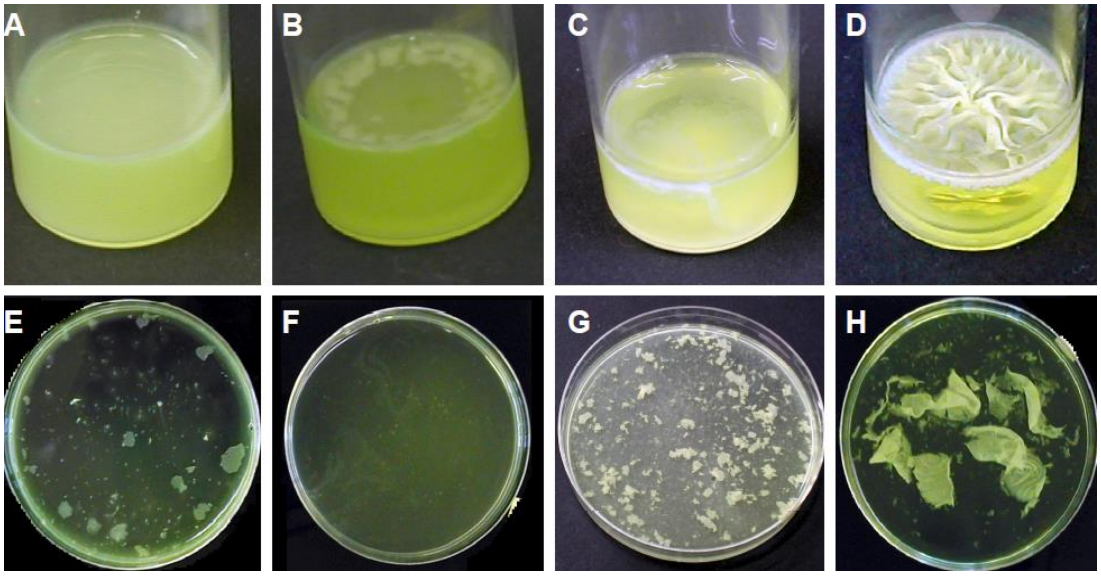


Figure 1.2 Shown are the phenotype of air-liquid (A-L) interface biofilms produced by *Pseudomonas* isolates from a 3-day-old cultures. (A) Uniform surface of a waxy aggregate biofilm (evolved *P*. *putida* PH6); (B) viscous mass biofilm (evolved from *P*. *aeruginosa* PAK); (C) floccular mass biofilm (evolved from River *Pseudomonas* sp. Isolated W2 Dec 18); and (D) an extreme example of a physically cohesive biofilm (evolved *Pseudomonas gessardii* CFML 95-251); and below are the material from the corresponding biofilms after their tipping into Petri dishes (Source: Spiers *et al.,* 2006; Ude *et al.,* 2006).

1.4.3 The Pseudomonads

The *Pseudomonas* genuses are Gram-negative, rod shaped bacteria including a number of species capable of consuming a wide range of organic and inorganic compounds, and lives under diverse environmental conditions (Margesin & Schinner, 2001). Thus, they are found in soil and water ecosystems etc. and as plant, animal and human commensals and pathogens (Griffin *et al*., 2003; Berg *et al*., 2005). The genus *Pseudomonas* confers a wide range of metabolic adaptability and genetic flexibility (Silby *et al*., 2011). They grow rapidly and have the ability to metabolize a broad range of substrates, including toxic organic chemicals, such as aliphatic and aromatic hydrocarbons

(Esmaeil & Akbar, 2015; Jha *et al*., 2015). *Pseudomonas* species also exhibit resistance towards antibiotics, disinfectants, detergents, heavy metals, and organic solvents (Dziewit *et al*., 2015; Jilani, 2015). Some strains have been established to produce metabolites that promote plant growth or inhibit plant pests (Ingham, 2011). Members of the genus are known to form biofilms (Robertson *et al*., 2013).

Pseudomonas species have been important agents for biotechnological applications, primarily due to varied genomes supporting a diverse range of metabolisms, and a tendency to allow genetic manipulation (Barbe *et al*., 2004). When used as a consortium, strains of numerous species of *Pseudomonas* are able to harness energy from an extensive range of relatively inert compounds (Hackbarth *et al*., 2014). The best studied amongst the species are the *Pseudomonas aeruginosa* known as an opportunistic human pathogen, the soil bacteria *Pseudomonas putida*, the plant growth promoting *Pseudomonas fluorescens* and the plant pathogen *Pseudomonas syringae*.

Pseudomonas aeruginosa are mostly found in soil, water, plants, humans, animals, wastewater, sewage, plants and in hospital equipment (Leclerc *et al*., 2002). This microorganism is capable of breaking down a wide range of organic molecules including organic compounds such as benzoate (Bhawsar & Singh, 2014).

Pseudomonas putida are found in most soil and water habitats where aeration persists. *P. putida* has many strains including the rhizosphere strain KT2440, the endophytic strain W19, the aromatic hydrocarbon- degrading strain F1 and the manganese-oxidizing strain GB-1 (Compant *et al*., 2010; Wu *et al.,* 2011).

The bacterium derives its nutrients through the rhizosphere of the plant, thereby supporting its survival and in return, the *P. putida* encourages plant growth and protects the plants from pathogens (Rodríguez-Navarro *et al*., 2007). Because *P. putida* assist in promoting plant improvement, it is used in bioengineering research to develop biopesticides and to advance plant health (Espinosa-Urgel *et al.,* 2000).

Pseudomonas fluorescens are found in soil, plants and water surfaces. They exist as commensal species with other plants allowing plants to maintain important nutrients, degrading pollutants, produced antibiotics that suppress pathogens. An in-depth study of this species has exposed its potentiality and beneficial use in bioremediation activity (Shim & Yang, 1999; Juhasz & Naidu, 2000; Barathi & Vasudevan, 2001).

Pseudomonas syringae are plant pathogen that is identified by an inability to utilize arginine, because it is not supported by the arginine dihydrolase system (Phillips, 2012). This species of bacteria is actually represented by over 50 different pathovar strains, which is a set of bacterial strains sharing similar features differentiated by their individual pathogenicity toward one or more plant hosts (Sarkar & Guttman, 2004). Each strain of *P. syringae* is specific for a particular plant (Morris *et al*., 2008). All *P. syringae* strains are deficient in a specific cytochrome C oxidase in their respiratory electron transport system, which results in a negative oxidase reaction (Manzoor, *et al*., 2012). It colonizes different surfaces including damaged plant tissues and leaves surfaces (Hofius *et al*., 2007).

On a general note, the interest in *Pseudomonas* bacteria is because they produce surface active compounds called biosurfactants. Many *Pseudomonas* species are implicated in surfactant production, although the type and nature of surfactant produced by each organism differ from one species to another (Ron & Rosenberg, 2002; Uzoigwe *et al*., 2015).

1.5 Bacterial surfactants

1.5.1 A brief introduction to surfactants and biosurfactants for the non-chemist

Surfactants are amphiphilic compounds that lower the existing free energy of a system by substituting it with combined molecules that exhibit higher energy at an interface. Due to their ability to capture contaminants, they are normally applied in soil washing and flushing systems (Pennell *et al*., 1994). Surfactants have been used in industries as adhesives, wetting and foaming agents, fluctuating, deemulsifiers and penetrants (Mulligan & Gibbs, 1993). In the petroleum industry surfactants are required to increase the solubility of oils especially in the process of enhanced oil recovery (Singh *et al*., 2007; Fakhru'l-Razi *et al*., 2009). Surfactants are also been used as emulsifiers in the pharmaceutical and cosmetic industries (de Araujo *et al*., 2014). The presence of surfactant in a system signifies that less amount of energy is needed to transfer the molecule to the surface, while the surface tension is generally reduced (Somasundaran & Huang, 2000). Surfactants that are capable of lowering the surface tension of water from 72 to 35 $mN.m^{-1}$ and the interfacial tension of water/hexadecane from 40 to 1 $mN.m^{-1}$ are generally referred to as

good surfactants (reviewed by Fakruddin, 2012). The strength of the surface active compound in a system is a direct measure of the surface tension before the critical micelle concentration (CMC) is reached. The lower the critical micelle concentration (CMC) the less surfactant is required to lower surface tension to the lowest level (at the CMC) (Herdes *et al*., 2015).

Surfactants are applied to treat hydrocarbon contaminated soils and water to improve the bioavailability of the hydrophobic organic compound thereby increasing their rate of degradation. A schematic diagram of surfactant-enhanced bioremediation of organic contaminated soils is shown in Figure 1.3.

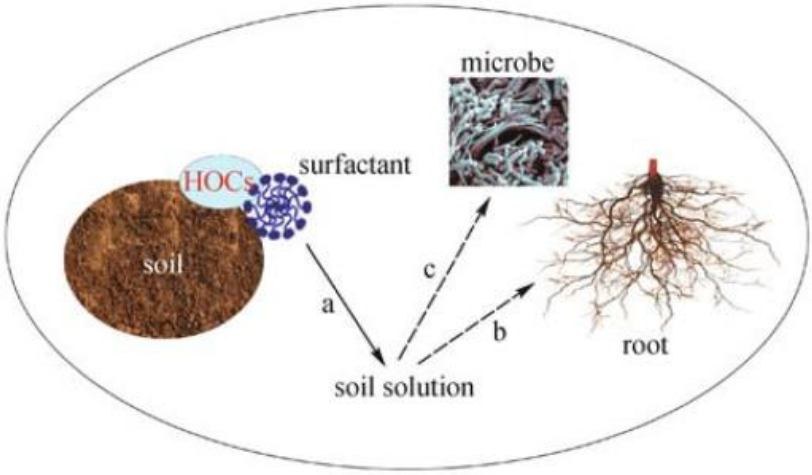


Figure 1.3 Schematic diagram of surfactant mediated bioremediation. Surfactants applied to soils contaminated by hydrophobic organic compounds (HOC), enhanced the bioavailability and utilization of the contaminants. (a) The addition of surfactant to HOC in soil dissolves it into soil solution, which is easly accessed through (b) the roots of plants and (c) the uptake of hydrophobic organic contaminants by microorganisms mobilized in soil solution i.e. bioremediation. (Source: Zhang & Zhu, 2014).

1.5.2 Types of biosurfactants produced by microorganisms and their uses

The main classes of biosurfactants include glycolipids, lipopeptides (e.g. surfactin, viscosin, fengycins, and iturins), phospholipids and polymeric biosurfactants. The best known glycolipids are rhamnolipids, sophorolipids and trehaloselipids (Makkar & Rockne, 2003; Yin *et al*., 2009; Sarubbo *et al*., 2014). The chemical structures of some common biosurfactants are shown in Figure 1.3. The diversity of biosurfactants is an important feature that allows these compounds to be used in a wide range of industrial and biotechnological applications, including agriculture, medicine, cosmetics, food processing, pharmaceutical and petroleum industries. The effectiveness of any surfactant produced by microorganisms may largely depend on how far it can reduce the surface tensions of liquids. A summary of biosurfactants producing organisms and their applications are shown in Table 1.2.

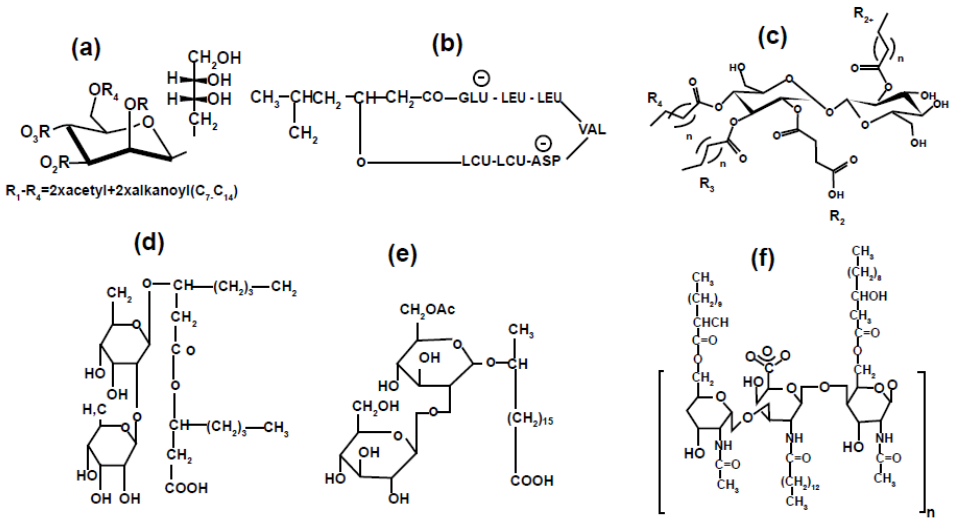


Figure 1.4 Shown are chemical structure of some common biosurfactants (a) Mannosylerythritol lipid produced by smut fungi *Ustilaginomycetes* (b) Surfactin produced by *Bacillus subtilis* (c) Trehalose lipid produced by *Rhodococcus erythropolis* (d) Sophorolipid produced by *Candida (Torulopsis) bombicola* (e) Rhamnolipid produced by *Pseudomonas aeruginosa* (f) Emulsan produced by *Acinetobacter calcoaceticus* (Source: Fakruddin, 2012).

Table 1.2 Biosurfactants, producing organisms and their applications

Microorganism	Types of biosurfactant	Applications	References
Rhodococcus erythropolis 3C-9	Glycolipid and Trehalose Lipid	Oil spill cleaning operations	Peng *et al*. 2007
Pseudomonas aeruginosa S2	Rhamnolipid	Bioremediation of places contaminated by petroleum	Chen *et al.* 2007
Pseudozyma slamesis CBS 9960	Mannossylthritol lipid	Promising yeast biosurfactant	Morita *et al.* 2008a
Pseudozyma graminicola CBS 10092	Mannossylthritol lipid	Washing detergent	Morita *et al.* 2008b
Pseudomonas libanesis M9-3	Lipopeptide	Remediation of metal-contaminated waste streams and soils and as antibiotics in medicine	Saini *et al.* 2008
Bacillus subtilis ZW-3	Lipopeptide	Pharmaceutical, environmental protection, cosmetics and petroleum recovery	Wang *et al.* 2009b
Rhodococcus sp TW 53	Lipopeptide	Bioremediation in sea environment	Peng *et al.* 2008
Pseudozyma hubeiensis	Glycolipid	Bioremediation in sea environment	Fukuoka *et al.* 2008
Rhodococcus wratislaviensis BN 38	Glycolipid	Environmental uses such as bioremediation of hydrocarbon contaminated soils	Tuleva *et al.* 2008
Bacillus subtilis BSS	Lipopeptide	Bioremediation in places contaminated by hydrocarbon	Abdel-Mawgoud *et al.* 2008
Azotobacter chroococcum	Lipopeptide	Environmental uses such as in oil pollution removal or bioremediation	Thavasi *et al.* 2008
Pseudomonas aeruginosa BS20	Rhamnolipid	Bioremediation in places contaminated by hydrocarbon	Abdel-Mawgoud *et al.* 2009
Micrococus luterus BN56	Trehalose tetraester	Bioremediation in places contaminated by petroleum	Tuleva *et al.* 2009

Bacillus subtilis HOB2	Lipopeptide	Petroleum recovery, bioremediation of soil and sea environments and food industries	Haddad *et al.* 2008
Pseudomonas aeruginosa UFPEDA 614	Rhamnolipid	Bioremediation	Neto *et al.* 2009
Nocardiopsis alba MSA10	Lipopeptide	Bioremediation	Gandhimathi *et al.* 2008
Pseudoxanthomonas sp. PNK-04	Rhamnolipid	Environmental uses such as hydrocarbon biodegradation	Nayak *et al.* 2008
Pseudozyma parantarctica	Mannossymannitol lipid	Detergent or washing emulsifiers	Morita *et al.* 2009
Pseudomonas alcaligenes	Rhamnolipid	Environmental uses such as removal of oil and petroleum contamination	Oliveira *et al.* 2009
Pseudomonas koreensis	Lipopeptide	Biocontrol agent	Hultberg *et al.* 2008
Pseudomonas fluorescens BDS	Lipopeptide	Bioremediation and Biomedicine	Janek *et al.* 2010
Candida bombicola	Sophorolipids	Environmental uses such as bioremediation	Daverey & Pakshirajan, 2010a,b
Brevibacterium aureum MSA 13	Lipopeptide	Petroleum recovery	Kiran *et al.* 2010b
Nocardiopsis lucentencis MSA04	Glycolipid	Bioremediation in sea environment	Kiran *et al.* 2010a
Bacillus velezensis H3	Lipopeptide	Industrial strain for lipopeptide production	Liu *et al.* 2010
Calyptogena soyoae	Mannossylerrythritol lipid	Bioremediation in sea environment	Konishi *et al.* 2010
Burkholderia plantari DSM 9509	Rhamnolipid	Pharmaceutical and detergent industries	Hormann *et al.* 2010

(Source: Makkar *et al.* 2011)

1.5.3 Prediction of minimum surface tension achieved by bacteria

In order to characterize the biosurfactants produced by microorganisms, a number of assays can be used. These includes emulsion assays, to measure emulsification index (E_{24}) as described by Cooper (1987) and also the hexadecane emulsification activity (E_{hex}) determined by Toren *et al*., (2001), and the standard emulsification assay (Rosenberg *et al*., 1987). Surfactants could also be characterized through cell surface hydrophobicity (Rosenberg *et al*., 1980) and contact angle measurements as described by Henriques *et al*. (2002). The critical micelles concentration (CMC) determined by turbiditmetric titration (Abed *et al*., 2004), and the surface tension measurement using tensiometers after Fechtner *et al*. (2011).

Recently, Individual distribution identification (IDI) analysis was then used to determine the minimum surface tension achievable by bacterial surfactants by fitting theoretical probability distributions, and selecting the best of fit using the Anderson-Darling goodness of fit test to predict the minimum liquid surface tension reducing activity (γ_{Min}) of bacterial cultures and was found to be 24.16 $mN.m^{-1}$ (Fetchner *et al.,* 2011). However, it remains to be seen how rigorous this prediction is, and whether it remains true for other collections of bacteria including soil isolated pseudomonads.

1.5.4 Biosurfactant-oil behaviour

Surfactant behaviour can be characterized using a number assays to measure the effects a surfactant has on oils present in the system. A summary of these assays are shown in Table 1.3.

A commonly oil used for biosurfactants behaviour assays is Used Lubricating Oil (ULO). These are spent oils that have been used in automobile engines to lubricate moving parts of engines in order to reduced frictions to the acceptable level and protect the system against wear and also facilitates the removal of contaminants the engine thereby acting as a cleaning, anticorrosive and cooling agents (Assunção Filho *et al.,* 2010; Abro *et al.,* 2013).

The oil collects some impurities and other component associated with engine wear. These includes metal particles (i.e. iron, steel, copper, lead, zinc etc.) and compounds such as barium, sulphur, water, dirt, burnt carbon, and ash, some of which are highly toxic in nature and result in harmful effects when discharged into the environment (Abro *et al.,* 2013). The composition of used lubricating oils also includes poly aromatic hydrocarbons (PAH), naphthalines, volatile PAH, semi-volatile PAH and alkanes.

Table 1.3 Summary of surfactant oil behaviour assays and quantification procedures

Assay	Quantification procedure	Reference
Emulsification activity	One unit of emulsification activity was defined as the amount of emulsifier that changed the emulsion absorbance.	Bosch *et al*., (1998) and Willumsen and Karlson (1997).
Emulsification stability	The absorbance was taken every 10 min for 50min. The log of the absorbance was plotted against time and its slope was taken to measure the stability of emulsions.	Prieto *et al*., 2008
Atomised oil assay	The assay can detect an array of different synthetic and bacterially produced surfactants. Lower concentration of many surfactants can be detected compared to the drop collapsed assay.	Burch *et al*., 2010
Contact angle measurements	Contact angles were measured using the sessile drop technique. The angles gotten on the surfactant films were used to calculate the total surface tension.	Henriques *et al*., 2002
Hemolytic activity	This was done by screening isolated strains on blood agar plates comprising 5% (v/v) blood and incubated for 24-48 h. The presence of clearing zone around a colony signifies Hemolytic activity	Carrillo *et al*., 1996
Oil spread technique	The diameter observed due to the effect of the biosurfactant relates to the strength of the clearing zone and this is measured as the concentration of the biosurfactant.	Morikowa *et al*., amd Youssef *et al*., 2004.
Stalagmometric method	This method operates on the principle that measures the weight of the drops of the fluid dropping from the capillary glass tube.	Chichkanoov *et al*., 2002.

1.7 Aims of this thesis

1.7.1 Study rationale

The main aims of the research presented in this Thesis were to investigate the diversity of surfactants produced by pseudomonad isolates through behavioural assays and to identify isolates for potential biotechnology applications in future studies.

A lot of current research has focused on the isolation, identification and characterization of single or small groups of biosurfactant producing microorganisms. This approach limits the rate at which new biosurfactants might be discovered, but it is unclear whether greater numbers can be found by screening larger samples, as biosurfactant variety may be limited. In order to investigate this possibility, a collection of pseudomonads was isolated and characterized, with those expressing biosurfactants determined by drop collapse assay and reported in Chapter Three of this Thesis.

Current research lists biosurfactant producing microorganisms and the liquid surface tension reducing ability of each, with the best subject to further analysis. This approach may be very inefficient as earlier work has shown by statistical means that there is a limit (γ_{min}) to biosurfactant activity (Fechtner *et al.,* 2011). This limit is tested using the biosurfactant producing pseudomonads as the major aim of the work reported in Chapter Four of this Thesis.

Microbial diversity is an interesting parameter because it is suggested that bacteria have a range of substrate utilization and metabolic activities, which

might influence surfactant production and behaviour in bioremediation, or the large scale isolation of biosurfactants in an industrial setting. A range of growth and plate based assays have been used to phenotype the pseudomonad isolates here to determine strain diversity (Chapter Three), whilst a range of biosurfactant assays including oil displacement, foaming and emulsion tests have been used to assess biosurfactant behaviour (i.e. structural diversity) in Chapter Five of this Thesis.

Finally, the biosurfactant producing pseudomonads isolated in this work were also assessed for their ability to produce biofilms, as the formation of this type of microbial aggregation is a key to colonization of surfaces such as those formed in soils, beaches and sediments where bioremediation is required, and is reported in Chapter Six of this Thesis.

1.7.2 Research objectives for this work

Isolation and characterisation of Pseudomonas spp*. bacteria (Chapter 3)*

1. To isolate pseudomonads from activated sludge wastewater and soil samples from a SUDS site;
2. To phenotypically characterise these isolates using a series growth-based and behaviour assays to assess diversity within the collection;
3. To identify those isolates likely to produce biosurfactants by drop-collapse assay for further investigation.

Pseudomonad surface tensions (γ) and the prediction of γ_{Min} (Chapter 4)

1. To determine the surface tension of cell-free culture supernatants produced by the drop-collapse positive pseudomonads by quantitative tensiometry;
2. To identify those pseudomonads significantly lowering surface tension by statistical means (the low-γ isolates);
3. To investigate the effect of incubation period and the presence of antagonistic compounds on the surface tension of cell-free culture supernatants; and
4. To predict the minimum surface tension γ_{Min} reducing activities of these isolates and to compare γ_{Min} with earlier work and published surface tension reports.

Diversity in Surfactant Behaviour (Chapter 5)

1. Determine whether the set of 41 low-γ (LSTRA - low surface tension reducing activity) isolates represents a diverse collection of strains using the phenotype data;
2. Determine the water and oil-water behaviours of surfactants produced by the low-γ LSTRA pseudomonads, using foam stability, emulsion and oil-displacement assays;
3. Identify a sub-set of isolates suitable for future chemical analysis and testing using correlation and clustering analysis of behavioural data; and
4. Investigate whether there is evidence to support chemical variation within surfactants expressed by sub-sets of isolates producing very similar surface tensions.

Characterization of biofilms produced by Pseudomonas spp*. in experimental microcosms (Chapter 6)*

1. To investigate and quantitatively characterise biofilm formation at the air-liquid interface in static microcosms, including assaying for cellulose as the primary EPS;
2. To compare biofilm formation in static microcosms with growth in a column bead system;
3. To determine whether biofilm formation occurs at the oil-water interface.

Chapter 2

Materials and methods

2.1 General Microbiology

2.1.1 Media and culturing conditions

Media used for bacterial growth are provided in Table 2.1 (media used for specialised assays, plus additives including antibiotics, etc. are listed in the following sections). Agar plates were made with 1.5% (w/v) Technical agar (Oxoid) unless otherwise specified and media were sterilised by autoclaving at 121°C for 20 minutes.

Incubations and assays were conducted at 20 ± 1°C unless otherwise stated. Inocula was either colony material sampled from fresh plates using a loop, or aliquots of over-night shaken KB cultures incubated in a Stuart Orbital Incubator S150 bench top shaker operating at 150 rpm. Experimental microcosms were sterilised 30 mL universal glass vials (Fisher Scientific, UK) containing 6 mL media after Rainey and Travisano (1998). Strains were maintained at -80°C in KB with 15% (v/v) glycerol. Strains were recovered from the -80°C stocks by streaking onto the appropriate selective plates before use. All experiments were conducted in replicates of three ($n = 3$), unless otherwise stated

Table 2.1 Media used for culturing bacteria

Media	Composition, Reference / Source
King's B (KB) medium	10 g glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_20$ and 20 g Proteose peptone No. 3 (Becton, Dickinson and Company, UK) per litre (King's *et al*., 1954).
Luria-Bertani (LB) medium	10g Tryptone, 10g Yeast extract and 10g NaCl per litre (Sambrook *et al*., 1989).
Minimal M9 medium	6 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 20 mM glucose per litre (Sambrook *et al*, 1989).
Nutrient medium	Supplied by Oxoid.
PSA (*Pseudomonas* selective agar)	*Pseudomonas* selective agar with CFC (centrimide, fusidin and cephaloridine) supplements (Oxoid).
Milk plates	2% Milk powder, 0.15% w/v Yeast extract (Oxoid) and 1.5% w/v Technical agar (Oxoid).
Tributryin plates	5g Tributryin agar plus 1, 2, 3-Trybutyrin glycerol (Sigma-Aldrich, UK).

2.1.2 Growth and other measurements by spectrophotometry

Growth was assessed by optical density (OD_{600}) using a Spectronic Helios Epsilon spectrophotometer (Thermo Fisher Scientific, UK) with 10 mm optical-path cuvettes. Spectrophotometry was also used for absorbance (A) measurements.

2.1.3 Bacterial strains used in this work

Bacteria used in this work are listed in Table 2.2 below

Table 2.2 Bacteria used in this work

Strain	Genotype / Description	Source
***P. fluorescens* SBW25**	Wild-type *Pseudomonas fluorescens* SBW25; expresses surfactant (viscosin) and produces cellulose-based biofilms.	Rainey & Bailey (1996)
P. fluorescens* SBW25 Δ*viscA	SBW25 but Δ*viscA*; does not express viscosin.	de Brujin *et al*. (2007)
***P. fluorescens* SS101**	Wild-type *Pseudomonas fluorescens* SS101, expresses surfactant (massetolide A).	de Souza *et al*. (2003)
P. fluorescens* SS101 Δ*massA	SS101 but Δ*massA*; does not express massetolide A.	de Brujin *et al*. (2008)
Wastewater Isolates	Strains recovered from wastewater (#1 - #252).	This work
Soil Isolates	Strains recovered from soil samples (#253 - #355).	This work

2.2 Sample collection

2.2.1 Wastewater

Three wastewater samples were collected monthly (11th April 2012, 10th May 2012 and 12th June 2012) from the activated sludge chamber of the Hatton

Wastewater Treatment Plant near Arbroath in 1 litre sterile plastic bottles and stored overnight at 4 °C before use.

2.2.2 Soil

Soil samples were collected from a Sustainable Urban Drainage System (SUDS) site with history of hydrocarbon contamination (Swale 9 Outlet; Appendix A1.1; SNIFFER Final Report, 2008) on the 13th September 2012. Samples were obtained from three different positions defined by the apexes of a triangle with 1 m sides and 10 cm below the surface and underneath the grass roots. At each position, three approximately 200 g samples were collected and then mixed to produce a single sample which was stored overnight at 4°C before use. A 100 g aliquot of each mixed sample was sent for chemical analysis, including arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), zinc (Zn), oil (Total Petroleum Hydrocarbon), orthophosphate (P), and ammoniacal nitrogen (N) (Environmental Scientific Group (ESG), UK), and the results are presented in Appendix A1.2.

2.3 Isolation of pseudomonads

Wastewater and soil samples were re-suspended (0.1 mL / 0.1 grams per 0.9 mL) in sterile de-ionised water. Serial dilutions were spread on semi-selective PSA-CFC plates for 72 h to isolate *Pseudomonas* and pseudomonad-like bacteria. Randomly-chosen colonies were re-streaked on PAS-CFC plates before being used to inoculate shaken over-night KB cultures which were then

stored at -80°C as glycerol stocks. A total of 355 isolates were preserved and recorded as strains #1 – #252 (Wastewater isolates) and #253 – #355 (Soil isolates) (Table 2.2).

2.4 Phenotypic characterisation

Bacteria isolated from wastewater and soil samples were characterised using a number of growth assays and biochemical tests as outlined in the following sections. Strains were assessed as being either positive or negative according to the description given for the expected outcomes of these assays. Positive and negative controls were not specifically used, as sufficient variation amongst the collection of isolates allowed a clear identification of results (all assays have been used to assess phenotypes previously and where appropriate, reported concentrations or conditions have been used here).

2.4.1 Acid production in the presence of maltose

Acid production was assessed after Robertson *et al.* (2013). Nutrient plates containing 20 $mg.L^{-1}$ phenol red and 10 mM maltose were drop-inoculated with 5 µl of over-night KB culture. Colonies which showed a colour change from pink to yellow were recorded as positive and those which remained pink were recorded negative.

2.4.2 Catalase test

Catalase activity was determined following the method described by Robertson *et al.* (2013), in which 50 µl of over-night KB culture was added to 10 µl of 30 % (v/v) hydrogen peroxide placed on the clean surface of a Petri dish lid. Mixtures that produced bubbles within 10 s were recorded as positive and those that did not were recorded as negative.

2.4.3 Drop collapse assay

Surfactant expression was assessed using the drop collapse assay as described by Chen *et al*. (2007). 10 µl drops of over-night KB culture were placed onto clean surface of a Petri dish lid and visual observation was made of the drops. A positive result was recorded if the droplet spread out across the lid surface and negative when the droplet kept its initial bead-like shape.

2.4.4 Gelatinase activity

Gelatinase activity was assessed after Robertson *et al.* (2013), using Nutrient medium plates containing 1.6% (w/v) gelatine (Customer Care-Baking Ltd, UK) instead of agar and drop-inoculated with 5 µl of over-night KB culture. A positive result was recorded if the region around the inoculation site was liquidised within 2 – 3 days and negative if the region remained solid.

2.4.5 Kanamycin resistance

Kanamycin resistance was assessed using LB plates containing 50 µg.mL^{-1} kanamycin after Champion *et al.* (2011). The plates were drop-inoculated with 5 µl of a 1:100 dilution of over-night KB culture and incubated for 2 days. If the growth of colonies was apparent, it was recorded as been resistant to the level of antibiotic used. If not it was recorded as not resistant (sensitive).

2.4.6 KB culture acidity

Acidity of KB cultures was assessed after Robertson *et al.* (2013) by preparing 0.025 g of Bromocresol green in 25 ml deionised water and adding a 5 µl drop of this solution to 50 µl of over-night KB culture. A positive result was recorded if the colour was dark blue (i.e. pH > 7) and a negative result if the colour is colourless or yellow.

2.4.7 Lipase activity

Lipase activity was assessed following the method described by Robertson *et al.* (2013). Tributyrin plates were drop-inoculated using 5 µl of over-night KB culture. A positive result was recorded if ring of clearance (halo) around the inoculated area after 1 - 2 days was observed. A negative result was recorded if the inoculated area showed no halo.

2.4.8 Mercury resistance

Mercury resistance was assessed using LB plates containing 10 µg.ml^{-1} of mercury chloride ($HgCl_2$), after (Kafilzadeh & Mirzaei, 2008). The plates were drop-inoculated with 5 µl of 1:100 dilution of overnight KB culture and incubated at room temperature for 48 hrs. If the growth of colonies was apparent, it was recorded as been resistant to the level of mercury chloride used. If not it was recorded as not been resistant (sensitive).

2.4.9 Oxidase test

Oxidase activity was determined after Robertson *et al.* (2013), by adding 10 µl of 1% TMPD (N, N, N', N'-tetramethyl-p-phenylenediamine) to colonies grown on KB plates. A positive result was recorded if blue/purple colour developed within 10 s and negative if changes did not occur within this time.

2.4.10 Protease secretion

Protease activity was assessed after Robertson *et al.* (2013), using milk powder plates drop-inoculated with 5 µl of over-night KB culture. A positive result was recorded if colonies showed a ring of clearance (halo) around the inoculation site after 1 – 2 days. The strains which showed no halo were recorded as negative.

2.4.11 Sodium chloride tolerance

Sodium chloride tolerance was assessed after (Robertson *et al.,* 2013) using LB plates containing 8% (w/v) NaCl, drop-inoculated with 5 µl of over-night KB culture. A positive result was recorded if colony growth occurred after 3 days and no growth was recorded as negative.

2.4.12 Swarming motility

Swarming motility was assessed after Robertson *et al.* (2013) using 0.5% agar plates (KB agar with 0.5% w/v agar) drop-inoculated with 5 µl of over-night KB culture. A positive result was recorded if irregular extensions of the colony edge were visible after 1 – 2 days and a negative result was recorded if the strain showed no irregular extension of the colony edge.

2.4.13 Sugar utilisation

Sugar utilization was assessed after Robertson *et al.* (2013) using Minimal M9 plates containing 20 mM glucose or sucrose solution drop-inoculated with 5 µl of over-night KB culture. A positive result was recorded if colony growth occurred after 3 days incubation and no growth the result was recorded negative.

2.4.14 Swimming motility

Swimming motility was assessed after Robertson *et al.* (2013) using stab-inoculated 0.3% agar plates (0.1x normal KB nutrients and 0.3 % w/v agar), with over-night KB culture. A positive result was recorded if a ring of expansion through the soft agar was observed after 1 – 2 days and growth was recorded negative if it was restricted to the stab mark.

2.4.15 Tetracycline resistance

Tetracycline resistance was assessed following the method described by Luo & Farrand, (1999). Luria-bertani (LB) agar plates containing 10 $\mu g.ml^{-1}$ of tetracycline were drop-inoculated with 5 µl of 1:100 dilution of over-night KB culture. If the growth of colonies was apparent, it was recorded as been resistant to the level of antibiotic used. If not it was recorded as not resistant (sensitive).

2.4.16 Twitching motility

Twitching motility was assessed after Robertson *et al.* (2013). This was stab-inoculated using pipette tips through a thin layer of 1.0 % agar plates (KB with 1.0% (w/v) agar) to the bottom using over-night KB culture. A positive result was recorded if an expanding zone of growth was observed between the Petri dish and agar interface, and negative if no growth was seen at the interface.

2.5 Biofilm formation

2.5.1 Qualitative assessment of biofilm-formation in static microcosms

Biofilm-formation in replicate static KB microcosms (*n* = 3) was investigated qualitatively after three days according to the biofilm classes described by Ude *et al.* (2006). Growth was observed by the turbid nature of liquid column, and biofilm-classes were assessed by inspection *in situ* and of the material produced when tipped into Petri dishes.

2.5.2 Quantitative assessment of biofilm-formation in static microcosms

To investigate growth, biofilm strength and attachment levels in static microcosms, combined biofilm assay were used (Robertson *et al*., 2013). Replicate KB microcosms (*n* = 8) were incubated statically for 3 days before assay. Strength (grams) was determined by maximum deformation mass (MDM) assay (Ude *et al*., 2006) in which glass balls (mean weight of 0.0115 g) were added until the biofilm broke or sank (a significant MDM was recorded when more than one replicate retained at least one ball for at least 5 s). Microcosms were then emptied into another vial and vortexed for 30 s before growth was determined by measuring the optical density (OD_{600}). The original microcosm vials were then washed three times in water with vigorous shaking, stained with 1 ml of 0.05% (w/v) Crystal violet for 2 min before washing three times again in water. The stain was eluded by shaking with 5 ml of 95 % ethanol for 2 h, and the level of attachment determined by measuring the absorbance (OD_{570}) of this solution (Spiers *et al*., 2003).

2.5.3 Glass bead column biofilms

Biofilm-formation was also assessed in partially-saturated glass bead columns after Udall *et al.* (2015). Replicate microcosms (*n* = 3) were constructed using autoclaved P5000 pipette tips containing a plug of glass wool followed by a 5 cm bed-depth of 1 – 2 mm diameter glass balls. These were put into racks placed in larger plastic boxes containing a beaker of water to control humidity and prevent drying-out. The microcosm were inoculated with 3 ml of over-night KB culture and allowed to drain freely to produce a partially-saturated porous network, and incubated for 3 days. These were destructively sampled by decanting the glass balls into vials containing 6 ml KB broth, vortexing for 30 s before growth was determined by measuring the optical density (OD_{600}).

2.6 Measurement and prediction of liquid surface tension

2.6.1 Measurement of liquid surface tension

Liquid surface tension (γ) of cell-free culture supernatants were measured with a KRÜSS K 100 Mk 2 Tensiometer (Krüss, Germany) using the standard rod method at 20°C after Fechtner *et al*. (2011). Using this method, γ of de-ionised water was 73.2 ± 0.1 mN m^{-1} and sterile KB medium was 52.8 ± 1.4 mN m^{-1}. Replicate over-night KB cultures (*n* = 4) were centrifuged at 4000 x g for 10 min to produce cell-free supernatants and the surface tension of the supernatant was measured.

2.6.2 Prediction of the minimum surface tension (γ_{Min})

In order to predict the minimum bacterial LSTRA (γ_{Min}), γ data from 50 LSTRA isolates were investigated. Data were examined by ANOVA, and *post hoc* multiple comparison Tukey-Kramer HSD (TK-HSD) test used to determine a set of 50 isolates that formed a single homogeneous class (α = 0.05) (IBM SPSS Statistics for Windows, Version 21.0. IBM Corp., USA). Individual distribution identification (IDI) analysis, based on the Anderson-Darling (AD) goodness of fit test, was then used to identify theoretical probability distributions to fit γ data and predict γ_{Min} using the threshold parameters of the fitted distributions (MINITAB v.15, Minitab Ltd, UK).

2.7 Cellulose expression

Cellulose expression was assessed qualitatively using Calcofluor plates and fluorescent microscopy after Spiers *et al.* (2003). Calcofluor plates were LB plates containing 10 µg Calcofluor ml^{-1} (Fluorescent Brightener 28; Sigma) and were inoculated with 5 µl of over-night KB cultures. The plates were viewed using an Alphamager HP system with a ML-26 UV transilluminator (GRI, UK) after 24hr incubation. A positive result was recorded if high-intensity fluorescence was observed and negative if not. Colony material was stained with 10 µg ml^{-1} Calcofluor for 1 h before inspection with a Leica DMR fluorescence microscope. A positive result was recorded if cellulose fibres were visible at 10x or 40x magnification and negative if not.

2.8 Microbial attachment to hydrocarbons (MATH) assay

The relative hydrophobicity (H_r) of cells was determined using the MATH assay after Rosenberg *et al.* (1980). Over-night KB cultures (*n* = 3) were vortexed with 3 mL Hexadecane for 5 s and allowed to stand for 20 min, before the OD_{6OO} of the aqueous phase was measured (OD_{600i}). The mixture was vortexed again for 60 s and left to stand for 20 min, before the OD_{600} was re-measured (OD_{600f}). The ratio of OD_{600f} / OD_{600i} gave the relative hydrophobicity of cells for each strain tested.

2.9 Emulsion assay

The relative indices of oil, aqueous and emulsions were determined using an emulsion assay (Prieto *et al*., 2008). This was done by mixing 5ml of autoclaved used lubricating oil (ULO) heated in water bath at 30°C for 30 min with 500 µl KB medium and bacterial culture supernatant (*n* = *3*). These were vortexed for 2 min and allowed to stand for 24 h. The emulsion index, E_{24}, was calculated using the equation E_{24} = 100 x Height of the emulsion layer (mm) / Total height (mm). The relative oil (O_i), aqueous (A_i), and emulsion (E_i) indices were also measured.

2.10 Oil-displacement assay

This test was conducted as described previously by Morikawa *et al*. (2000). 40 ml of distilled water was put in replicate petri-dishes (*n* = 3) and 10µl of sterile

used lubricating oil (ULO) was placed to form a thin layer at the surface. Culture supernatant (10µl) was gently dropped at the centre of the oil layer. If surfactant was present in the culture supernatant, the oil would be displaced to produce an oil-free zone and the diameter of the zone (mm) was measured within 5 s. If no surfactant was present, no zone would be formed.

2.11 Bacterial foaming assay

Stability of foams was measured following the method used by Sathe and Salunke (1981) with modifications. Over-night KB cultures in replicates (n = 3) were vortexed for 30 second to generate foam. The initial height (H_i) of foam was measured (mm), and then allowed to stand for 2 h after which the height was re-measured as the final height (H_f). The reduction in foam heights (foam reduction) were determined from the equation, percentage (ΔH) = $100(H_i - H_f) / H_i$.

2.12 Statistics

Statistical analyses used Graphpad (www.graphpad.com/quickcals/), JMP v7.0 (SAS Institute Inc., USA), MINITAB v15 (Minitab Ltd, UK), (IBM SPSS Statistics for Windows, Version 21.0. IBM Corp., USA). Differences between means were investigated by ANOVA, Tukey–Kramer HSD and t-tests assuming unequal variances. Phenotype and surfactant behaviour data was examined by Hierarchical cluster analysis using the Ward method without any weighting of factors (phenotypes, i.e. each factor were treated equally) (JMP 7.0).

Independence between surface tension and phenotype data or surfactant behaviour data were tested using a *2 x 2* contingency table-approach and significance amongst groups was tested using the Fisher's Exact test (Graphpad). The prediction of the lowest surface tension reducing activity followed the approach of Fechtner *et al*. (2011) using Individual distribution identification (IDI) analysis (MINITAB). Means with standard errors (SE), *P* values and replicate size are provided where applicable.

Chapter 3

Isolation and characterisation of *Pseudomonas* spp. bacteria

3.1 Introduction

Bacteria are widely distributed and abundant in the environment (reviewed by Nemergut *et al.,* 2011; Zhang *et al.,* 2013). Soils contain more than a billion bacteria per gram dry weight composing of thousands of different species (Wright, 2010; Goldfarb *et al.,* 2011).

The genus *Pseudomonas* (the pseudomonads) have been reported to be ubiquitous in nature and are Gram-negative, catalase and oxidase positive and can be found in soil, on plant material, and in water environments, as well as in human populations (reviewed by El-Benna, 2007; Calvayrac *et al.,* 2014; Rumbaugh, 2014).

Pseudomonas spp. are known to play significant roles in crop production to promote nutrient availability and growth, regulating the activities of plant pathogens and the production of antimicrobial agents (see reviews by Hayat *et al.,* 2010; Saranraj *et al.,* 2013; Zhou *et al.,* 2014).

More recently, Pseudomonads have been identified as the predominant group of soil microorganisms used to treat organic pollutants in soil and water, i.e.

bioremediation of contaminated soils (reviewed by Wasi *et al*., 2013; Basha & Rajaganesh 2014; Chen *et al.*, 2014). For example *P. putida, P. aeruginosa* and *P. fluorescens* strains has successfully been used in the laboratory to degrade petroleum-based products such as kerosene, gasoline, diesel oil and crude oil (Banat *et al.,* 2000; Abalos *et al.,* 2004; Rajasekar *et al.,* 2010).

In order to isolate pseudomonads from activated sludge wastewater, soil samples or other environmental samples, samples can be spread on PSA-CFC (centrimide, fusidin and cephaloridine) plates and incubated at 28°C under aerobic conditions (Arnaut-Rollier *et al.,* 1999; Purnell *et al.,* 2014). Randomly-chosen colonies of different morphologies were re-streaked for purity before further examination and identification.

Isolates were phenotypically characterized using a number of assays, including oxidase and catalase tests, swimming, swarming and twitching motilities, gelatinase, lipase, protease, and surfactant expression, sugar utilization and acid production in the presence of maltose. Mercury, kanamycin and tetracycline resistance tests can also be used to determine the levels of diversity as antimicrobial resistance can be quite variable amongst strains (Vaz-Moreira *et al.,* 2011; Islam *et al.,* 2013; Robertson *et al*., 2013). Pseudomonads can be further identified using metabolic profiling (e.g. API strips) or by 16S rDNA sequencing.

Although there are a number of interesting assays that can be used to phenotypically characterize isolates (e.g. lipase, protease, gelatinase activity,

sugar utilization) these tests can also inform applications including bioremediation where different growth requirements are important.

In order to screen isolates for biosurfactants expression, drop-collapse assays using cultures can be used before quantitative tensiometry of liquid cultures as described by Fechtner *et al*. (2011).

3.2 Research objectives

The aim of the research reported in this chapter was to isolate and characterise pseudomonads (i.e. *Pseudomonas* spp*.* and *Pseudomonas*-like bacteria) from activated sludge wastewater of a treatment plant and hydrocarbon-contaminated soils samples recovered from a Sustainable Urban Drainage Systems (SUDS) site for further investigation into surfactant expression, oil-behaviour and biofilm formation.

The objectives of this research were:

1. To isolate pseudomonads and *Pseudomonas*-like from activated sludge wastewater and soil samples from a SUDS site;

2. To phenotypically characterise these isolates using a series growth-based and behaviour assays to assess diversity within the collection;

3. To identify those isolates likely to produce biosurfactants by drop-collapse assay for further investigation.

3.3 Wastewater and soil samples for the isolation of pseudomonads

In order to isolate pseudomonads and *Pseudomonas*-like bacteria, two different sites were considered which might contain bacteria with potential to degrade hydrocarbons; the activated sludge wastewater from a treatment plant and soil samples from a sustainable urban drainage system (SUDS) site. Activated sludge wastewater contains bacteria, cooking oils from households, lubricating oils and other petrochemical products (Wagner *et al.,* 2002; Jameel *et al.,* 2011). Whereas the sustainable urban drainage system (SUDS) collects rainfall runoff from the road containing broad spectrum of contaminants i.e. traces of oil and fuel, chemical and rubber residues, biocides, metals from vehicle exhausts, resulting from mixing of wastewater from different sources (Graczyk *et al.,* 2008; Shakunthala *et al.,* 2010). In the past, most of the bacteria isolated for use in remediation activity had been collected from sites with high concentration of pollutants i.e. automobile workshops (Saimmai *et al.,* 2012; Ameko *et al.,* 2013; Bandyopadhyay *et al.,* 2014) but for this research, sites thought to have low or moderate concentrations of contaminants were considered.

The Hatton Wastewater Treatment Plant and the Sustainable Urban Drainage System (SUDS) site are shown in Figures 3.1 (A & B). Soil sample characterization (Appendix A1.1) produced additional environmental information which may be of use in future research into these strains.

Wastewater and soil samples were re-suspended in sterile de-ionised water and serial dilutions spread on semi-selective PSA-CFC plates to isolate pseudomonads. Randomly-chosen colonies were re-streaked on KB plates and examples of the individual colonies formed are shown in Figure 3.2.

Figure 3.1 Wastewater and SUDS Soil samples collection sites. Samples used to isolate *Pseudomonas* spp. bacteria were collected from activated sludge chamber of the Hatton Wastewater Treatment plant with a plastic bucket (A). Soil samples were collected from a sustainable urban drainage system (SUDS) sites on swale 9 outlet (B) of the A92 road, with a spade.

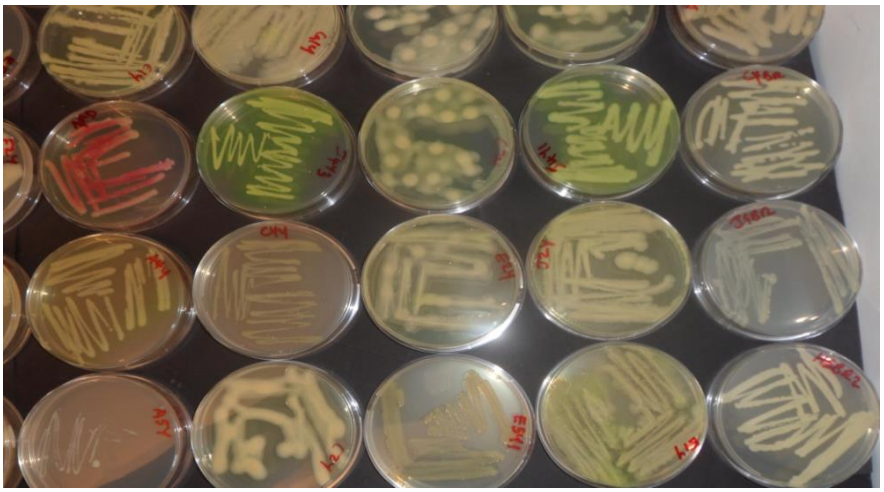


Figure 3.2 Re-streaked colonies of bacteria on KB Plates. Strains obtained from soil and wastewater environment are diverse as shown by considerable variation in colony colour.

3.4 Phenotypic analyses results

3.4.1 Phenotypic characterisation of isolates obtained from activated sludge wastewater and soils from a SUDS site

The phenotypic characterisation of pseudomonads from activated sludge wastewater data are listed in Tables A1.4 & A1.5 (Appendix A1). In summary, strains showed positive to 162 (64%) oxidase, 100 (40%) catalase and 116 (46%) siderophore tests. For motility tests, 155 (62%) swimming, 96 (38%) swarming and 51 (20%) twitching were positive. It was also found out from the test that 63 (25%) expressed surfactant, 75 (30%) lipase, 197 (78%) protease and 217 (86%) gelatinase secretion, 207 (82%) utilized sugar and 206 (82%) produced acid in the presence of maltose. Resistance to antibiotics and heavy metal where also tested and found 218 (87%) mercury, 220 (87%) tetracycline and 193 (77%) kanamycin to show positive to antibiotic resistance respectively.

The phenotypic characterisation of pseudomonads from SUDS soils data are listed in Tables A1.4 & A1.5 (Appendix A1). In summary, strains recorded positive for 81 (97%) oxidase, 9 (9%) catalase and 18 (17%) siderophore tests. For motility tests, we confirmed 29 (28%) swimming, 21 (20%) swarming and 9 (9%) twitching positive. The test showed 8 (8%) expressed surfactant, while secretion test indicates 51 (50%) lipase, 54 (52%) protease and 86 (83%) gelatinase positive, 90 (87%) sugar utilization capacity, 86 (83%) produced acid in the presence of maltose. Resistance to antibiotics and heavy metal where also tested and found 88 (85%) mercury, 87 (84%) tetracycline and 83 (81%) kanamycin resistance positive. None of the pseudomonad isolates responded

positive to KB culture acidity and sodium chloride tolerance tests for the wastewater and soil samples assayed.

3.4.2 Cluster analysis of phenotype data

In order to determine whether bacteria isolated from wastewater and SUDS soil are diverse collection of strains a Hierarchical cluster analysis (HCA) of the isolate phenotype data was undertaken after the method of Robertson *et al.* (2013). In these analyses all phenotypes were given equal weighting. The results of this (HCA) are shown in the dendrogram in Figure 3.3, and are clustered into 54 groups based on similarity as shown in Table 3.1. The clustering of surfactant positive and negative isolates based on the origin of isolates (wastewater or soil) is shown in Table 3.2. This analysis shows that this collection represents a diverse group of isolates with a minimal level of biological replication.

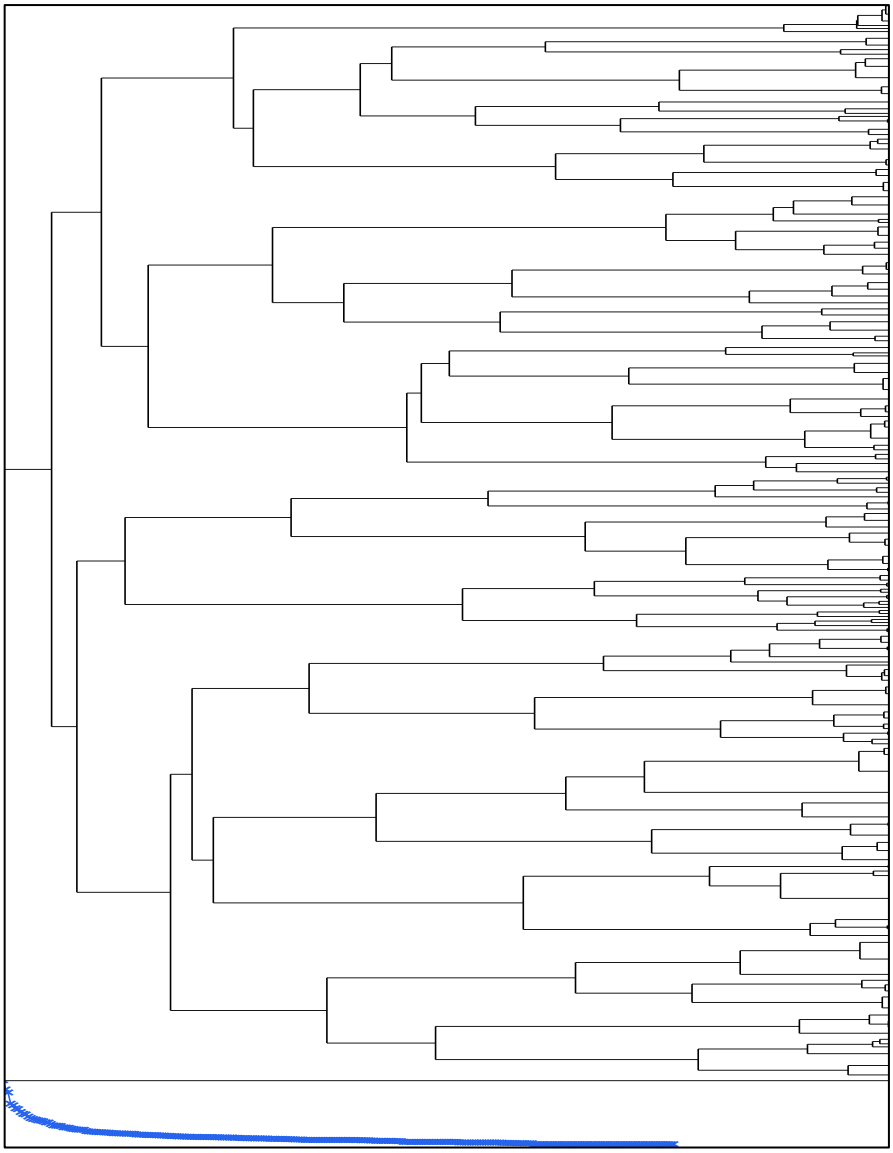


Figure 3.3 Pseudomonads isolates showed a diverse collection. Hierarchical cluster analysis of the phenotype data determined for the set of activated sludge wastewater and SUDS soil pseudomonads or *pseudomonas*-like bacteria demonstrates a high level of diversity. This dendrogram links isolates on the basis of similarity, and is shown with an even-spacing horizontal scale and a scree plot at the bottom where the ordinate shows the distance that was bridged to join the clusters at each step**.**

Table 3.1 Clustering of isolates obtained from wastewater and SUDS soil

Cluster groups	Isolates
1.	#1, #5, #8, #9, #63, #65, #67, #73, #79, #159
2.	#4, #11, #39,#122
3.	#15, #157, #253, #277, #322
4.	#16, #17, #18, #26, #266, #293, #335
5.	#260, #330, #339, #24, #124, #130, #207, #221, #222, #232
6.	#2, #69, #83, #108, #187
7.	#14, #60, #81, #354
8.	#21, #37, #43, #102, #188, #269
9.	#30, #143, #166, #171, #178, #269
10.	#29, #49, #154, #155, #156, #248, #252
11.	#3, #7, #10
12.	#72, #165, #185, #192, #306
13.	#27, #204, # 215, #218,#224
14.	#58, #97, #101, #179, #327, #336
15.	#20, #28, #44, #52, #345
16.	#23, #25, #36, #41, #209, #297
17.	#51, #142, #172, #225, #254, #305, #331
18.	#56, #77, #92, #100, #136, #167, #200, #283, #288, #289, #290, #301
19.	#57, #62, #64, #66, #96, #163, #168, #175, #183, #184, #195, #198
20.	#19, #38, #40, #349, #353
21.	#31, #135, #138, #149, #216, #255, #276, #298, #302, #334, #344
22.	#34, #116, #127, #284
23.	#70, #74, #93, #95, #99, #103, #176, #196, #199
24.	#256, #282, #304, #308, #313, #315, #317, #318, #319, #320, #321, #329, #341
25.	#46, #112, #125, #128, #139, #145, #220, #233, #235, #247, #249, #261, #270, #273
26.	#61, #94, #160, #279, #280
27.	#85, #88, #107, #111, #169, #174, #177, #193, #197, #228, #237
28.	#68, #80, #82, #84, #110, #180
29.	#115, #120, #123, #126, #129, #140, #148, #151, #201, #210, #241, #258, #265, #299, #311, #324, #338
30.	#117, #141, #147, #150, #153, #161, #173, #214, #257, #259, #262, #292, #316
31.	#6, #86, #211
32.	#32, #309, #348
33.	#55, #131, #203, #219, #227, #230
34.	#47, #343, #346, #347, #351, #355
35.	#12, #350, #352
36.	#59, #76, #326
37.	#114, #236, #239, #250, #342
38.	#226, #263, #268, #278, #294, #295, #296, #300, #314, #328
39.	#75, #105, #106, #189, #190
40.	#42, #45, #152
41.	#48, #50
42.	#87, #104, #158, #162, #170, #291
43.	#164, #182, #244, #267, #323
44.	#119, #133, #144, #208, #275, #286, #287, #303
45.	#121, #134, #202, #223, #272
46.	#78, #90, #118, #137, #146, #312, #340
47.	#191, #194, #238, #271
48.	#33, #53, #54, #231
49.	#91, #98, #109, #310
50.	#13, #35, #71, #89, #205
51.	#132, #206, #240, #242, #245, #246, #264
52.	#113, #212, #213, #217, #243
53	#181, #186, #274, #281, #307, #325, #332, #333, #337
54	#251, #285

The 355 isolates were clustered into 54 groups based on similarity using the Hierarchical cluster analysis by the Ward method. #, Wastewater isolates, #, SUDS soil isolates.

3.4.3 Screening for surfactant expression

This was achieved by drop-collapse assay of 18hr overnight KB cultures (after Persson & Molin, 1987). Drops of culture were placed onto the surface of a clean glass slide and a positive result was recorded if the droplet spread out across the surface and negative when the droplet retained its bead-like shape (as shown in Figure 3.4). Using this assay, a total of 71 (20%) strains were found to be expressing biosurfactants (listed in Table 3.2).

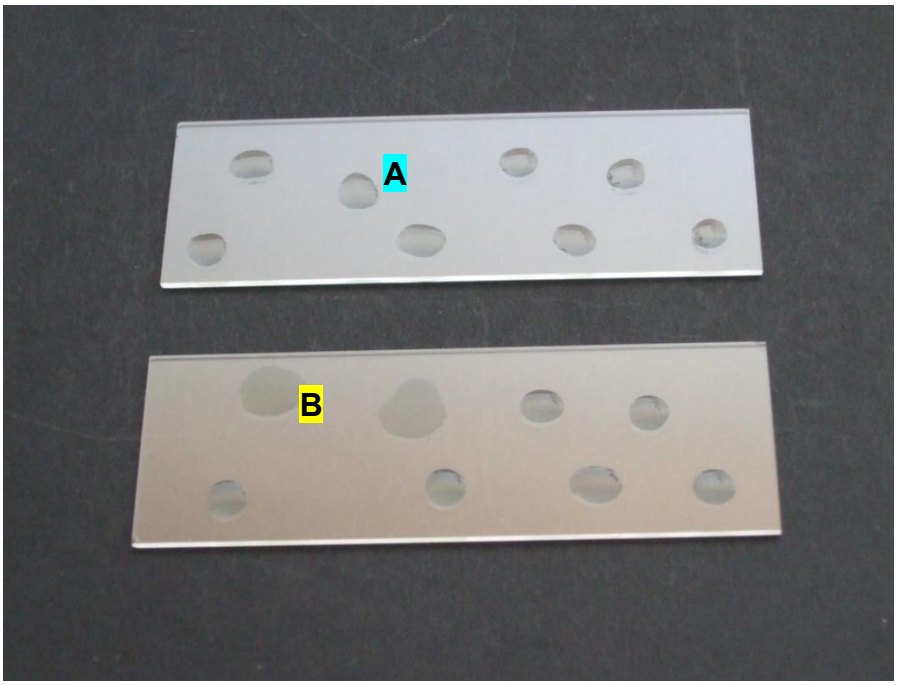


Figure 3.4 Drop collapse assay. Positive surfactant expression was assessed by investigating the surface tension of 18 hr KB cultures using the drop collapse assay. Drops of cultures were placed onto the surface of a clean glass slide. (A) Cultures not expressing surfactant produce beaded shapes indicating negative surfactant expression and (B) Cultures showing flattened drop with irregular edges indicates positive surfactant expression.

Table 3.2 Surfactant positive isolates recovered from activated sludge wastewater and SUDS soils

Origin	Biosurfactant positive Isolates
Wastewater	#1, #2, #5, #6, #8, #9, #14, #17, #42, #46, #47, #51, #58, #61, #63, #64, #65, #67, #69, #73, #75, #79, #81, #83, #85, #86, #88, #92, #94, #97, #101, #105, #106, #107, #111, #114, #152, #157, #159, #166, #167, #169, #174, #177, #178, #179, #183, #189, #190, #193, #194, #195, #197, #204, #208, #211, #228, #236, #237, #238, #239, #240, #250
SUDS	#280, #290, #303, #327, #336, #342, #354, #355

A total of 63 isolates from wastewater and 8 from SUDS soils were found to express biosurfactant from drop-collapsed test.

Table 3.3 Clustering of surfactant positive and negative isolates by Hierarchical cluster analysis of phenotype data

	Clusters of wastewater isolates	Clusters of soil isolates	Clusters of mixed isolates from wastewater	Clusters of mixed isolates from soil
Surfactant positive isolates	#1, #2, #5, #6, #8, #9, #42, #63, #64, #65, #67, #69, #73, #75, #79, #83, #85, #86, #88, #105, #106, #107, #111, #152, #159, #169, #174, #177, #183, #189, #190, #193, #195, #197, #204, #211, #228, #237.	None	#14, #46, #47, #51, #58, #61, #81, #92, #94, #97, #101, #114, #157, #166, #167, #178, #179, #194, #208, #236, #238, #239, #240, #250.	#280, #290, #303, #327, #336, #342, #354, #355.
Surfactant negative isolates	#3, #4, #7, #10, #11, #13, #21, #27, #29, #33, #35, #37, #39, #43, #45, #48, #49, #50, #53, #54, #55, #57, #58, #62, #66, #70, #71, #80, #84, #89, #93, #95, #96, #99, #102, #103, #108, #110, #113, #122, #131, #154, #155, #156, #163, #168, #175, #176, #180, #184, #187, #188, #196, #198, #199, #203, #205, #212, #213, #215, #217, #218, #219, #224, #227, #229, #230, #231, #243, #248, #252	#251, #256, #282, #285, #304, #308, #313, #315, #317, #318, #319, #320, #321, #329, #341.	#12, #15, #16, #18, #19, #20, #21, #22, #23, #24, #25, #26, #28, #30, #31, #32, #34, #36, #38, #40, #41, #44, #52, #56, #59, #60, #72, #76, #77, #78, #87, #90, #91, #98, #100, #104, #109, #124, #112, #115, #116, #117, #118, #119, #120, #121, #123, #125, #126, #127, #128, #129, #130, #132, #133, #134, #135, #136, #137, #138, #139, #140, #141, #142, #143, #144, #145, #146, #147, #148, #149, #150, #151, #153, #158, #160, #161, #162, #164, #165, #170, #171, #172, #173, #181, #182, #185, #186, #191, #192, #200, #201, #202, #206, #207, #209, #210, #214, #216, #220, #221, #222, #223, #225, #226, #232, #233, #235, #241, #242, #244, ss#245, #246, #247, #249.	#253, #254, #255, #257, #258, #259, #260, #261, #262, #263, #264, #265, #266, #267, #268, #269, #270, #271, #272, #273, #274, #275, #276, #277, #278, #279, #281, #283, #284, #286, #287, #288, #289, #291, #292, #293, #294, #295, #296, #297, #298, #299, #300, #301, #302, #305, #306, #309, #310, #311, #312, #314, #316, #322, #323, #324, #325, #326, #328, #330, #331, #332, #333, #334, #335, #337, #338, #339, #340, #343, #344, #345, #346, #347, #348, #349, #350, #351, #352, #353

3.5 Discussion

A total of 355 pseudomonads or *Pseudomonas*-like bacteria were recovered from activated sludge wastewater and SUDS soils. Pseudomonads are readily recovered from soil and rhizosphere samples using similar techniques including selection on PSA-CFC plates (Kwon *et al.*, 2005; Lakshmi *et al.*, 2014). In contrast, *Pseudomonas* spp. are rarely recovered from wastewater treatment plants, though they are present in detectable numbers (Ali & Naseem, 2012; Faldu *et al.,* 2014; Xu *et al.,* 2014).

The 355 isolates recovered on PSA-CFC plates were further examined using a series of phenotype assays, including catalase, oxidase, swimming motility and siderophore expression to show that the isolates were likely to be *Pseudomonas* spp*.* or *Pseudomonas*-like spp. Although the identities of these strains were not examined further (e.g. by 16S DNA sequence analysis), they are assumed to be all pseudomonads or *Pseudomonas*-like and are hereafter referred to as pseudomonads.

Screening for biosurfactant producing isolates by the drop collapsed assay, showed that 63 (25%) of the 252 isolates from wastewater and 8 (7.8%) of the 103 isolates are from the soil produce biosurfactants (Table 3.2).

Hierarchical cluster analyses of the 355 isolates using the phenotype data showed that this collection was diverse and are clustered into 54 groups based on similarity (Figure 3.3 & Table 3.1). Wastewater isolates clustered into 19 (35%) of the 54 groups, while the soil isolates clustered into 2 (3.7%) groups. The wastewater and soil isolates clustered into a mixed group of 33 (61%) of

the 54 groups (Figure 3.3 & Table 3.1). A total of 38 (34%) of the 111 isolates in wastewater clusters are surfactant positive, and 71 (63%) are surfactant negative, and all the 15 isolates in soil clusters are surfactant negative (Table 3.3). A total of 24 (10.5%) of the 228 isolates in mixed clusters from wastewater are surfactant positive, while 115 (50.4%) of the 228 isolates in mixed clusters from wastewater are surfactant negative (Table 3.3). 8 (3.5%) of the 228 mixed clustered isolates from the soil are surfactant positive, while 80 (35.1%) of the 228 mixed clustered isolates from the soil are surfactant negative.

Further characterization of these isolates could be achieved using metabolic profiling or 16S rDNA sequencing for which species identities could be obtained. However, in this work the isolates were not to be taxonomically determined to genus or species level, as the aim is to produce a collection of pseudomonads for further analysis of surfactant expression where variation would be interesting.

3.6 Conclusion

The research work presented in this chapter resulted in a collection 355 pseudomonads from activated sludge wastewater and SUDS soil for further investigation. This collection was diverse as shown by Hierarchical cluster analysis of isolate phenotype data, with few biological replications.

These isolates are screened for surfactant expression by the drop collapse assay, identifying 71 isolates (20%) likely to be expressing biosurfactant under

the conditions used here. These isolates are investigated further in Chapters Four, Five and Six of this Thesis.

Chapter 4

Pseudomonad surface tensions (γ) and the prediction of γ_{Min}

- *Sections of the text in this chapter are based on the manuscript submitted to Letters in Applied Microbiology (now published, Mohammed et al., 2015).*
- *The statistical analysis in this chapter was done in collaboration with Dr. Simona Hapca, a statistician with the SIMBIOS Centre (Now University of Dundee).*

4.1 Introduction

Biosurfactants are surface active chemicals expressed by a range of organisms that reduce liquid surface tensions (γ) of aqueous and aqueous-hydrocarbon (oil) mixtures. The biological roles for bacterial biosurfactants include motility and virulence, the inhibition of nematode and protist predation, lysis of fungi and oomycetes, and the induction of systemic resistance in plants (reviewed by Raaijmakers *et al*., 2010), as well as modifying water distribution in soil pore networks (Fechtner *et al*., 2011).

Bacterial biosurfactant expression is readily surveyed using modifications of the drop-collapse assay (Persson and Molin, 1987), blood agar, oil plates and oil sprays, and quantified by tensiometry of cultures or purified preparations (e.g. Bodour *et al*., 2003; Youssef *et al*., 2004; Burch *et al*., 2010). Surfactants can be behaviourally characterised by foaming, emulsion and oil-displacement

assays, and are known to be structurally diverse, including glycolipids, lipopeptides, lipopolysaccharides, etc. (Marchant *et al*., 2012; Ławniczak *et al.,* 2013; Sachdev and Cameotra, 2013).

The process from the isolation of biosurfactant expressing bacteria through to the chemical-structural analysis of biosurfactants is time consuming, and a recent evaluation of the liquid surface tension reducing ability (LSTRA) of environmental pseudomonads by Individual distribution identification analysis suggests that biosurfactant activity may be limited to a of minimum 24 mN m^{-1}(γ_{Min}) after Fechtner *et al*. (2011). Surface tension is inversely proportional to activity or strength. The first bacterial biosurfactant to be characterised, surfactin, expressed by *Bacillus subtilis,* could reduce γ to 27 mN m^{-1} (Peypoux *et al*. 1999). Perhaps surprisingly, since then significantly higher activities producing lower surface tensions have not been reported, despite the isolation and characterisation of many more biosurfactants from a range of different bacteria (e.g. 22 – 25 mN m^{-1} reported by Morikawa *et al.,* 1993; Nielsen *et al*., 2002; Kuiper *et al*., 2004; Fechtner *et al*., 2011; Xia *et al*., 2011; Saimmai *et al*., 2012). A limit to biosurfactant activity suggests a biological restriction in the synthesis of these agents or a need to prevent self-damage during expression (Fechtner *et al*., 2011). *In extremis*, surfactant absorption to the air-liquid interface may be kinetically limited, and under these conditions the physical-chemical properties of the solution and atmosphere will also be important.

4.2 Research Objectives

The aim of the research reported in this chapter was to investigate by quantitative tensiometry the drop-collapse positive pseudomonads recovered from wastewater and contaminated soil expected to express surfactants, and to use this data to predict the minimum surface tension that bacterial cultures might achieve when grown *in vivo*. This information would be helpful for the selection of isolates for future chemical analyses and testing in bioremediation and biotechnology.

The objectives of this research were:

1. To determine the surface tension of cell-free culture supernatants produced by the drop-collapse positive pseudomonads by quantitative tensiometry;

2. To identify those pseudomonads significantly lowering surface tension by statistical means (the low-γ LSTRA isolates);

3. To investigate the effect of incubation period and the presence of antagonistic compounds on the surface tension of cell-free culture supernatants; and

4. To predict the minimum surface tension γ_{Min} reducing activities of these isolates and to compare γ_{Min} with earlier work and published surface tension reports

4.3 Identification and classification of surfactant producing isolates

Surfactant producing strains were further characterized using overnight KB cultures centrifuged to provide cell-free culture supernatant as described by Fechtner *et al*. (2011). The surface tension data were analysed using individual distribution identification (IDI) analysis based on Anderson-Darling goodness of fit test, was used to identify a theoretical probability distribution to fit the surface tension data that was further used to predict the minimum surface tension (Fechtner *et al*., 2011).

4.3.1 Identification of surfactant producing isolates

In order to identify surfactant producing isolates by quantitative tensiometry. The 18 h cell free KB culture supernatant of isolates, likely to be expressing surfactants by the drop collapse assay (from Chapter Three) plus 14 randomly-chosen drop-collapse negative isolates were investigated further by quantitative tensiometry using a K100 Mk 2 Tensiometer (Krüss, Germany) by the rod method. Using this equipment the surface tension of water and KB were also measured (The mean surface tension data is shown in Appendix A2, Table A2.1).

A total of 57 of 85 isolates significantly reduced the liquid surface tension (γ_{min}) of sterile King's B (KB) broth medium from 52.8 ± 0.5 $mN.m^{-1}$ (dashed line) to 24.5 ± 0.1 – 49.1 ± 1.4 $mN.m^{-1}$ (Fig. 4.1), whilst the remaining 28 isolates did not (Dunnett's method using γ_{KB} as the control, α = 0.05). From left to right as

shown in Fig. 4.1, the LSTRA isolates are #69 – #75 and non-LSTRA isolates #114 – #311, respectively. The LSTRA isolates are further differentiated into two homologous groups, the low (#69 – #183) and intermediate (#5 – #75) surface tension groups using (TK-Kramer HSD, α = 0.05) as indicated in Fig 4.1.

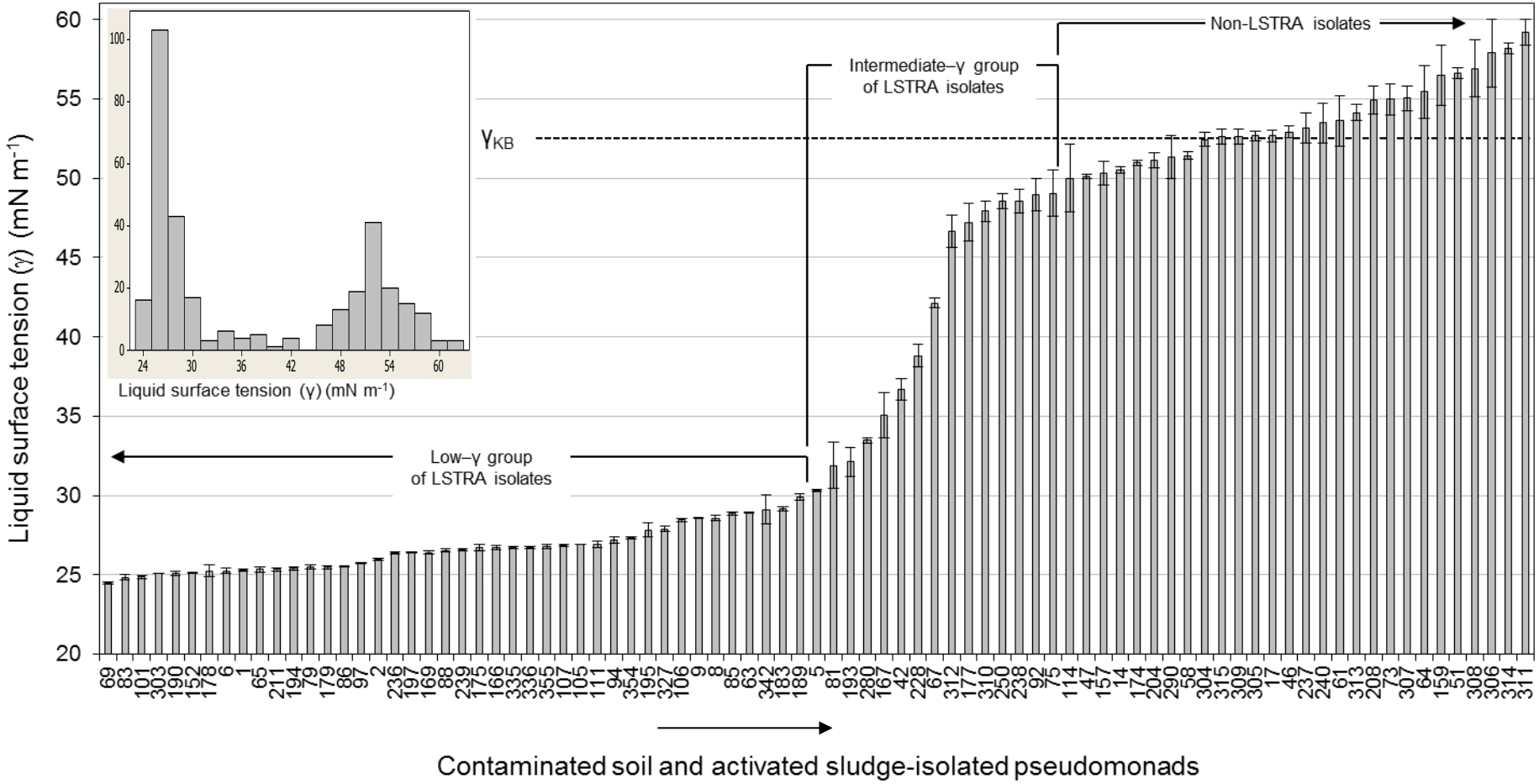

Liquid surface tension (γ) (mN m-1)
60
55
50
45
40
35
30
25
20
γKB
Non-LSTRA isolates
Intermediate–γ group
of LSTRA isolates
Low–γ group
of LSTRA isolates
100
80
60
40
20
0
24
30
36
42
48
54
60
Liquid surface tension (γ) (mN m-1)
69 83 101 303 190 152 178 6 1 65 211 194 79 179 86 97 2 236 197 169 88 239 175 166 335 336 355 107 105 111 94 354 195 327 106 9 8 85 63 342 183 189 5 81 193 280 167 42 228 67 312 177 310 250 238 92 75 114 47 157 14 174 204 290 58 304 315 309 305 17 46 237 240 61 313 208 73 307 64 159 51 308 306 314 311
Contaminated soil and activated sludge-isolated pseudomonads

Figure 4.1 (Previous page) Distribution of surface tension expressions of a collection of Pseudomonads and *Pseudomonas*-like bacteria. From left to right, LSTRA-isolates #69 – #75 and non-LSTRA isolates #114 – #311, respectively). The LSTRA isolates are further differentiated into the low and intermediate-γ groups as indicated. Liquid surface tension was determined by tensiometry of cell-free supernatants produced from 24 h shaken KB culture cultures. Mean and standard errors (n = 4) are shown. The bimodal distribution of surface tension measurements is shown in the inset histogram.

4.3.2 Concluding statement

A total of 85 pseudomonads or *Pseudomonas*-like isolates, comprising of 71 drop-collapse positive and a range of 14 colonies of the negative isolates were picked and investigated by quantitative tensiometry of culture supernatant to confirm the likely expression of surfactants. Of these 57 significantly reduced the surface tension (LSTRA isolates #69 – #75) and the remaining 28 isolates (non-LSTRA isolates #114 – #311) did not. The LSTRA isolates were further differentiated into the low-γ groups (isolates #69 – #183) and intermediate-γ groups (isolates #5 – #75) respectively.

4.4 Sensitivity of surfactant expression to environmental and culture conditions

Surfactant expression is known to be sensitive to culture conditions, including medium, length of incubation, temperature (Ghurye *et al.*, 1994; Bicca *et al.*, 1999; Fechtner *et al.,* 2011; Matvyeyeva *et al.*, 2014; Wadhwani *et al.*, 2014). Here the impact of extended incubation periods and the possible presence of surface antagonists were investigated.

4.4.1 Increased incubation period has little impact on surface tension

In order investigate whether the increase in incubation period affects surface tension. A comparison between 24 h and 48 h KB culture supernatants was done by randomly selecting 8 isolates from low, intermediate and non-LSTRA

groups. Liquid surface tension was measured for cell-free supernatants produced from replicate (n = 4) of 24 h and 48 h cultures. Significant growth increase between the 24 h and 48 h cultures was confirmed by OD_{600} measurements (t-test, $P < 0.0001$).

'High' and 'low' level expressions were found to be relative. Increases in surface tension may be attributed to culture aging, whilst decreases are attributed to increased surfactant expression. Only two of the eight isolates tested across the non-LSTRA, intermediate-γ and low-γ LSTRA groups showed significant but minor decreases in surface tensions of 2 – 5.3 $mN.m^{-1}$ ($P \leq 0.05$) (Table 4.2). It was also observed that, more often surface tension increased by 0.3 – 4.1 $mN.m^{-1}$ ($P \leq 0.05$), and this might be due to the effects of culture aging and cell lysis as reported by Fechtner *et al*. (2011).

Table 4.2 Differences in liquid surface tension due to increase in incubation periods from 24 h and 48 h.

		Liquid surface tension ($mN.m^{-1}$)			
Isolate No.	**Group**	**24 h**	**48 h**	***P* value**	**Explanation**
#51	Non-LSTRA	56.6 ± 0.3	55.6 ± 0.4	0.0915	No surfactant expressed
#307	Non-LSTRA	55.0 ± 0.8	59.1 ± 0.5	0.0076	No surfactant expressed
#228	Intermediate	38.8 ± 0.7	40.3 ± 0.5	0.1706	Weak surfactants expressed at high level
#42	Intermediate	36.5 ± 1.0	37.5 ± 0.9	0.5206	Weak surfactants expressed at high level
#167	Intermediate	35.1 ± 1.4	39.9 ± 1.1	0.0400	Weak surfactant expressed at high level
#81	Intermediate	31.9 ± 1.5	26.6 ± 0.1	0.0366	Strong surfactant expressed at low level
#85	Low	28.9 ± 0.1	26.9 ± 0.1	0.0001	Strong surfactant expressed at low level
#303	Low	25.1 ± 0.0	25.4 ± 0.1	0.0108	Strong surfactant expressed at high level

The Liquid surface tension of the 8 randomly selected isolates were investigated by tensiometry of cell-free 18 h KB cultures (n = 4), for increased in incubation time. Means and standard errors are shown.

4.4.2 No evidence of antagonistic compounds found

In order to investigate the presence of compounds that might interact and affect surfactant production, 18 h cell free supernatants of isolates were mixed together under the same conditions and the mean surface tensions of the individual isolates and their mixtures were measured. The results showed that there is no evidence of antagonism (Fig 4.2) between isolates (Tukey-Kramer-HSD).

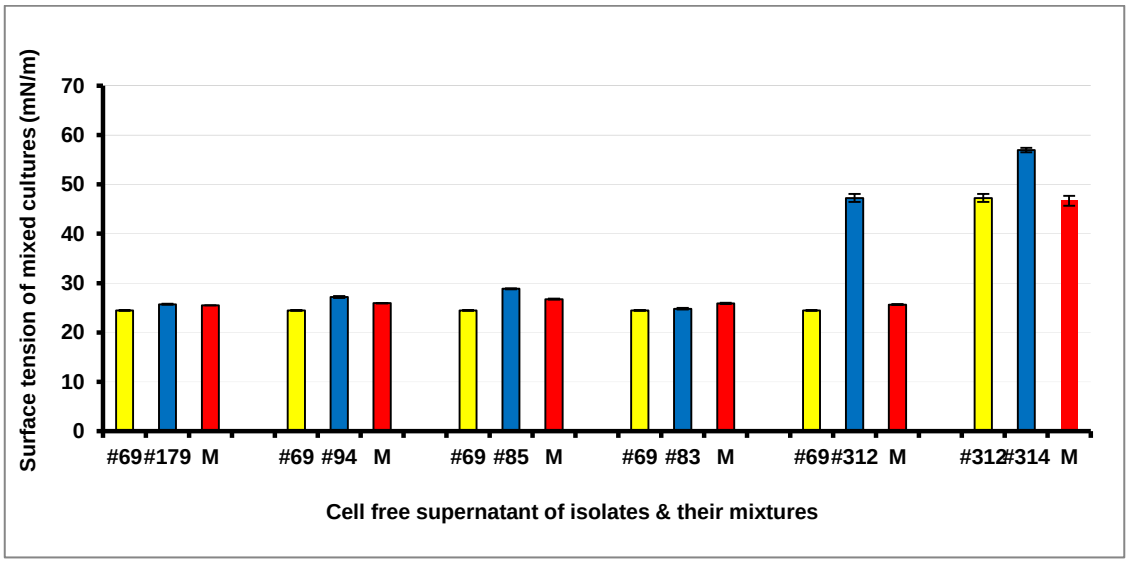


Figure 4.2 The presence of antagonistic compound was not found in the mixtures of low, intermediate and non-LSTRA isolates. This was carried out using replicate 18 h KB cultures (*n* = 4) with the mean ± SE of surface tensions of cell free supernatant of isolates and their mixture (M) shown. The result shows there is no antagonistic effect between individual isolates and their mixtures (TK-HSD, α 0.05).

4.4.3 Concluding statement

Increasing the incubation periods did not result in surfactant expression in non-LSTRA isolates as only minor decreases were observed in surface tensions. No significant differences were observed between surface tensions of individual isolates and their respective mixtures, indicating that no evidence found of antagonistic compounds between cell-free cultures of individual isolates and their mixtures.

4.5 Prediction of the minimum surface tension reducing activities of bacteria

Biosurfactant-producing bacteria have been surveyed in the past but only recently an attempt has been made to predict the minimum liquid surface tension these biosurfactant producing bacteria can achieve. Using the technique established by Fechtner *et al*. (2011), the tensiometry data were statistically analysed to determine a fundamental limit for biosurfactant activity in bacterial cultures incubated under standard growth conditions.

4.5.1 Prediction of the minimum bacterial biosurfactant activity (γ_{Min}) in cultures

In order to predict the minimum bacterial biosurfactant activity (γ_{Min}) in cultures, analysis of the surface tension data for 50 LSTRA isolates that formed a single large homogeneous group was undertaken (Isolates #69 - #67 as shown left to right in Figure 4.1), excluding the remaining 7 LSTRA isolates as they showed a

poorer ability to reduce surface tension and appeared statistically to be an extension of the non-LSTRA group (TK-HSD, $\alpha = 0.05$).

Individual distribution identification (IDI) analysis was used to determine the minimum surface tension from 16 theoretical distributions in MINITAB and found that gamma, log-normal and log-logistic distributions fitted the surface tension data well, with the best-fit provided by a log-logistic distribution based on the Anderson-Darling goodness of fit test (AD = 0.497, *P* = 0.293) predicting γ_{Min} of 24.24 $mN.m^{-1}$ as shown in Figure 4.4.

This estimated a γ_{Min} threshold value of 24.24 $mN.m^{-1}$, was also shown to be in agreement with log-normal and gamma predictions, adding confidence that the true LSTRA γ_{Min} for this collection of pseudomonads isolates is close to 24 $mN.m^{-1}$ as indicated in the three parameter distribution plots shown in Figure 4.5.

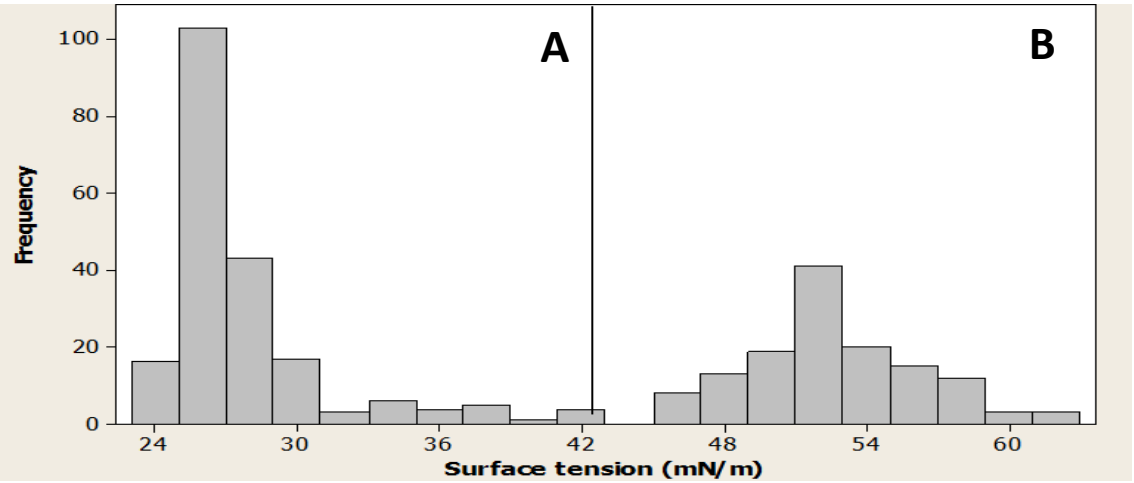


Figure 4.3 Distribution of surface tension data as informed by the homogeneous grouping table (Appendix A2.3). Shown is the frequency distribution of mean surface tension of all isolates. Section 'A' represent isolates with mean surface tension value ≤ 42.136 $mN.m^{-1}$ corresponding to isolate #67 truncated and used for this analysis.

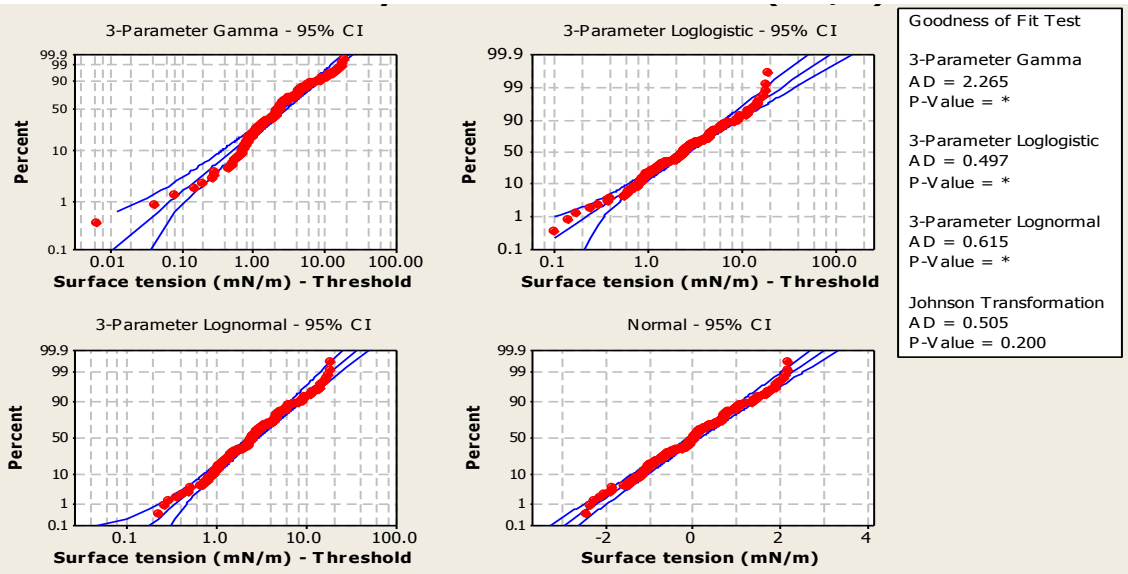


Figure 4.4 Probability plot for determining the minimum bacterial liquid surface tension (γ_{Min}) reducing activities. Shown are the 3-parameter distributions (gamma, log-normal and log-logistic as well as the Johnson transformation), in which γ_{Min} was limited from below by a threshold parameter to fit the data well, and the best being the log logistic distribution, as indicated by a low Anderson–Darling test statistic value (AD = 0.497, $P > 0.20$).

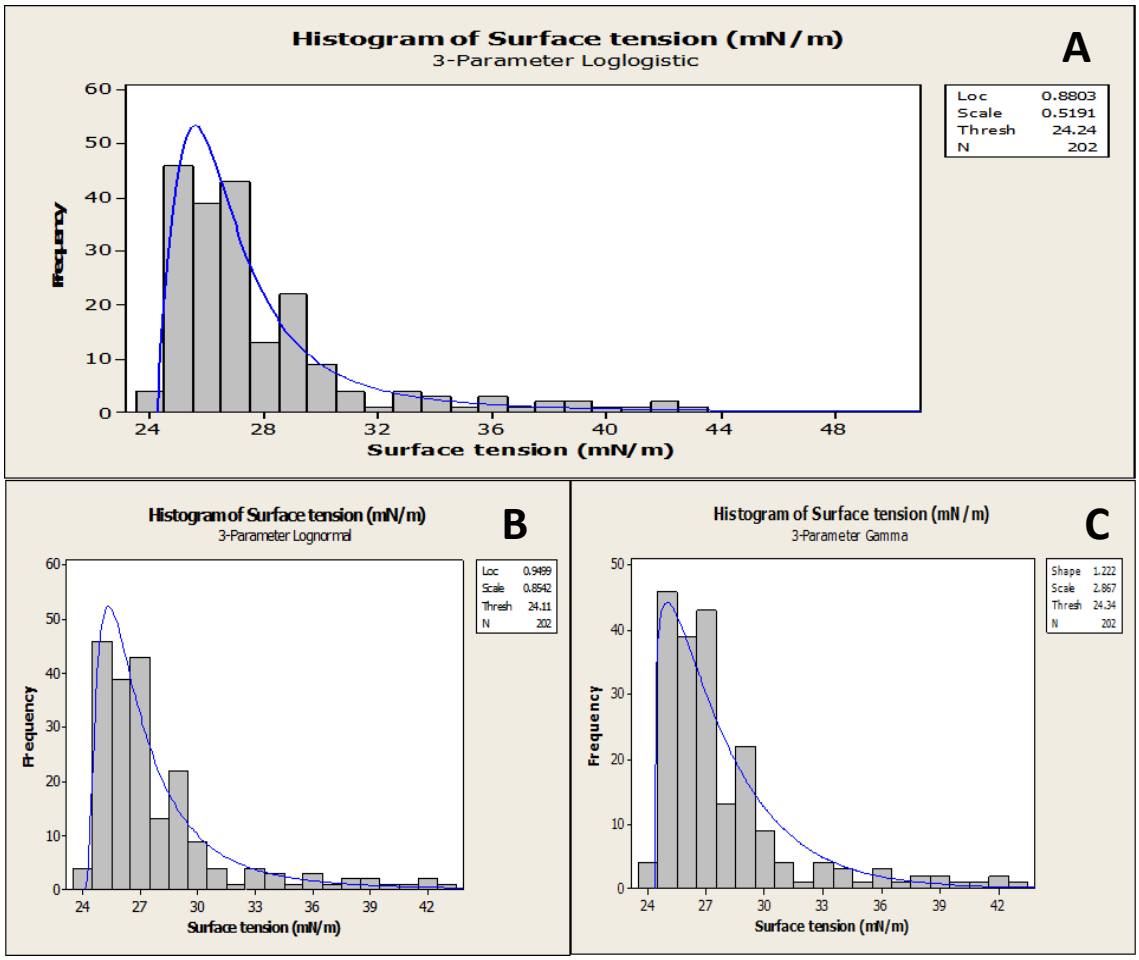


Figure 4.5 The frequency distribution curves predicting the minimum liquid surface tensions of bacteria producing surfactants can achieve. The threshold value of the minimum surface tension values are shown in all the three parameter curves with A (Loglogistics, 24.24 $mN.m^{-1}$), B (Lognormal, 24.11 $mN.m^{-1}$) and C (Gamma, 24.34 $mN.m^{-1}$). This figure was obtained from SPSS.

4.5.2 Prediction based on published reports of surface tension

In order to predict bacterial liquid surface tension reducing activity (LSTRA) from published reports, random selections of 59 mixed bacterial spp. with mean surface tension data ≤ 42.1mN.m^{-1} (Fig. 4.3) was made from a range of bacteria including non-pseudomonads from Table 4.3.

Individual distribution identification (IDI) analysis was used to determine the minimum surface tension by subjecting the published data to 16 theoretical distributions in MINITAB and found that log-normal, log-logistic, Gamma, Weibull and the one based on the Johnson transformation fitted the surface tension data well, with the best-fit provided by 3 parameter Weibull based on the Anderson-Darling goodness of fit test (AD = 0.238, $P > 0.500$) for which a γ_{Min} threshold value of 24.23 mN.m^{-1} was estimated (Fig. 4.4), which is in agreement with the finding of the current study.

Table 4.3 Bacterial liquid surface tension reducing activity (LSTRA) selected from published reports.

Isolate	Liquid surface tension (γ) ($mN.m^{-1}$)	Reference
Acinetobacter calcoaceticus BU03	38.4	Zhao & Wong (2009)
Aeribacillus pallidus YM-1	45.7	Zheng *et al.* (2012)
Bacillus amyloliquefaciens BZ-6	30	Liu *et al.* (2012)
Bacillus licheniformis JF-2	26.5	"
Bacillus sp. LAMI005	28.8	Sousa *et al.* (2012)
Bacillus sp. LAMI009	27.1	"
Bacillus subtilis ATCC 21332	27	Whang *et al.* (2008)
Bacillus subtilis DSVP23	26.5	Pemmaraju *et al.* (2012)
Bacillus subtilis HOB2	27	Haddad *et al.* (2009)
Bacillus subtilis LAMI008	38.1	Ponte Rocha *et al.* (2009)
Brevibacilis brevis HOB1	29	Haddad *et al.* (2008)
Burkholderia cepacia	45.7	Yalcin & Ergene (2010)
Dietzia maris WR-3	31	Nakano *et al.* (2011)
Geobacillus stearothermophilus UCP 986	31	Jara *et al.* (2013)
Klebsiella sp. RJ-03	44.06	Jain *et al.* (2012)
Lactobacillus paracasei	41.8	Gudiña *et al.* (2010)
Oleomonas sagaranensis AT18	25	Saimmai *et al.* (2012)
Paenibacillus alvei ARN63	35	Najafi *et al.* (2011)
Pseudomonas aeruginosa	35.9	Yalcin & Ergene (2010)
Pseudomonas aeruginosa BN10	29	Christova *et al.* (2011)
Pseudomonas aeruginosa Bs20	30	Abdel-Mawgoud *et al.* (2009)
Pseudomonas aeruginosa C450R	35.1	Mnif *et al.* (2011)
Pseudomonas aeruginosa CPCL	44	Arutchelvi & Doble (2010)
Pseudomonas aeruginosa DS10-129	27.9	Rahman *et al.* (2010)
Pseudomonas aeruginosa J4	29	Whang *et al.* (2008)
Pseudomonas aeruginosa L2-1	30	Costa *et al.* (2010)
Pseudomonas aeruginosa LBI	32	Pirôllo *et al.* (2008)
Pseudomonas aeruginosa MA01	32.5	Abbasi *et al.* (2013)
Pseudomonas aeruginosa MM1011	26	Amani *et al.* (2013)
Pseudomonas aeruginosa MR01	28	Lotfabad *et al.* (2009)
Pseudomonas aeruginosa MTCC2297	31.3	George & Jayachandran (2009)
Pseudomonas aeruginosa MTCC7815	35	Bordoloi & Konwar (2009)
Pseudomonas aeruginosa NY3	32.8	Nie *et al.* (2010)
Pseudomonas aeruginosa OBP1	31.1	Bharali & Konwar (2011)
Pseudomonas aeruginosa OCD1	31.5	Sahoo *et al.* (2010)
Pseudomonas aeruginosa PACL	26	de Lima *et al.* (2009)
Pseudomonas aeruginosa SP4	30	Pornsunthorntawee *et al.* (2009)
Pseudomonas aeruginosa UCP0992	27.4	Silva *et al.* (2010)
Pseudomonas aeruginosa WJ-1	24.5	Xia *et al.* (2012)
Pseudomonas fluorescens	28.4	Stoimenova *et al.* (2009)
Pseudomonas fluorescens	51.6	Yalcin & Ergene (2010)
Pseudomonas fluorescens 8573	27	Hvozdiak *et al.* (2009)
Pseudomonas fluorescens BD5	31.5	Janek *et al.* (2010)
Pseudomonas nitroreducens	37	Onwosi & Odibo (2012)
Pseudomonas putida	49.2	Yalcin & Ergene (2010)
Pseudomonas sp. 2B	30.14	Aparna *et al.* (2012)
Pseudomonas sp. BP10	48.46	Kumari *et al.* (2012)
Pseudomonas sp. RB91F	35	Belcher *et al.* (2012)
Pseudozyma aphidis	27	Recke *et al.* (2013)
Rhodococcus fascians A-3	27	Gesheva *et al.* (2010)

Rhodococcus sp. NJ2	65.24	Kumari *et al.* (2012)
Rhodococcus sp. TA6	30	Shavandi *et al.* (2011)
Selenomonas ruminantium CT2	25.5	Saimmai *et al.* (2013)
Shewanella sp. RB91G	54	Belcher *et al.* (2012)
Sphingobacterium sp.	32	Burgos-Díaz *et al.* (2011)
Staphylococcus sp. 1E	25.9	Eddouaouda *et al.* (2012)
Stenotrophomonas maltophilia RB91B	52.7	Belcher *et al.* (2012)
Streptomyces species B3	29	Khopade *et al.* (2012)
Strain 1	35	Youseef *et al.* (2004)
Strain 2	36.7	“
Strain 3	44	“
Strain 4	45.7	“
Strain 5	47.5	“
Strain 6	50	“
Strain 7	51	“
Strain 8	52	“
Strain 9	52	“
Strain 10	54	“
Strain 11	60	“
Strain 12	60.5	“
Strain 13	64	“
Strain 14	65	“
Strain 15	67	“
Strain DSVP2	28.72	Pemmaraju *et al.* (2012)
Strain DSVP9	29.65	“
Strain DSVP11	32.58	“
Strain DSVP18	30.24	“
Strain S3	34.8	Bozo-Hurtado *et al.* (2012)
Strain S10	33.9	“
Strain S19	36.1	“
Strain S21	41.7	“
Strain S29	37.2	“

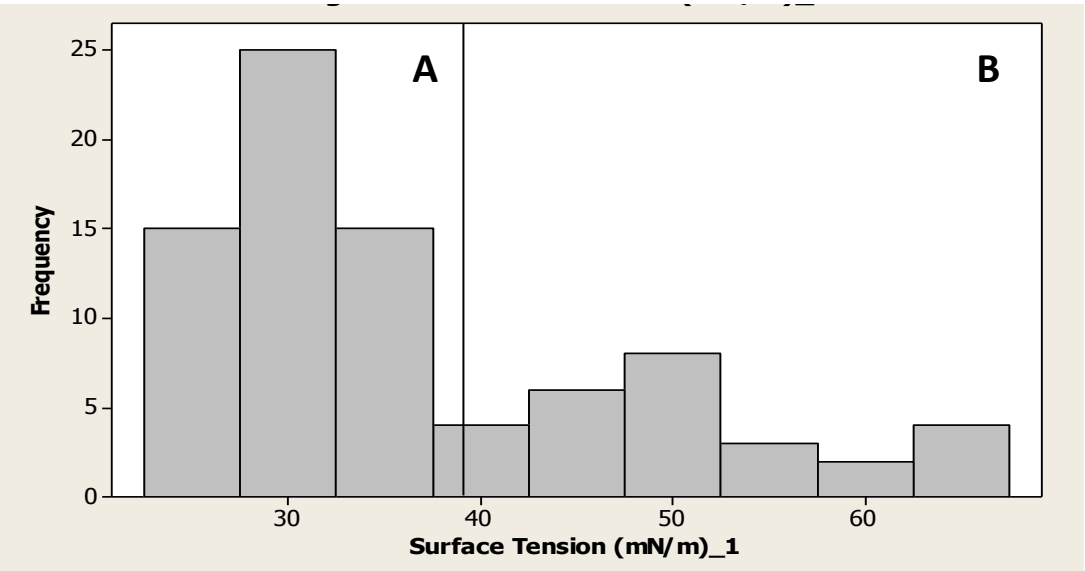


Figure 4.6 Distribution of surface tension data as informed by the homogeneous grouping table (Appendix A2.3). Shown is the frequency distribution of mean surface tension of random selections of the 59 mixed bacterial spp. Section 'A' represent the mixed bacterial spp. with mean surface tension value ≤ 42.136 $mN.m^{-1}$, truncated and used for this analysis.

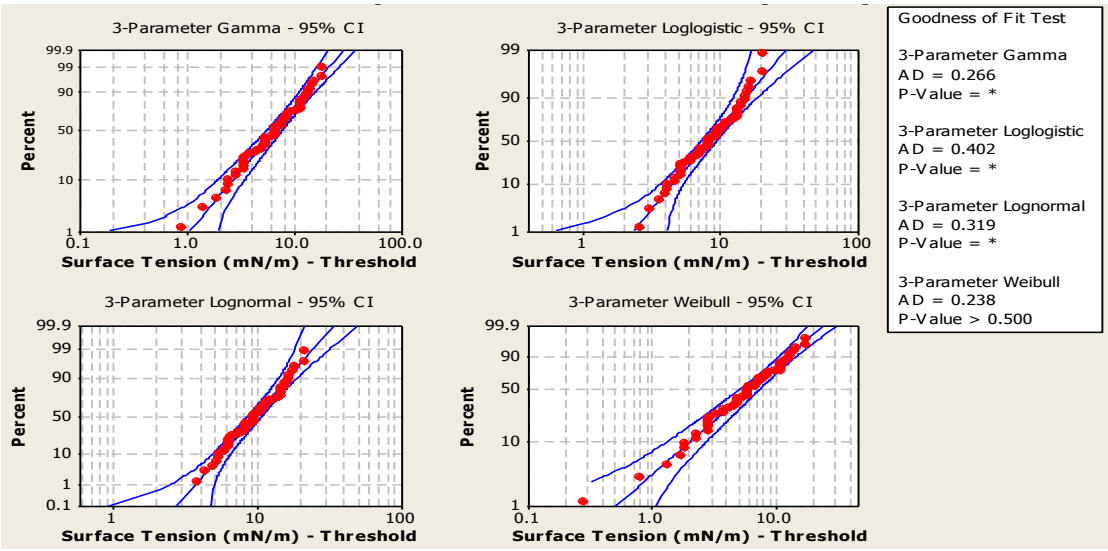


Figure 4.7 Probability plot for determining the minimum bacterial liquid surface tension (γ_{Min}) reducing activities of random sampling of published reports of mixed bacterial spp. Shown are the 3-parameter distributions (log-normal, Weibull, log-logistic and Gamma as well as Johnson transformation), in which γ_{Min} was limited from below by a threshold parameter to fit the data well, and the best being the 3-parameter Weibull, as indicated by a low Anderson-Darling test statistic value (AD = 0.238, $P > 0.500$).

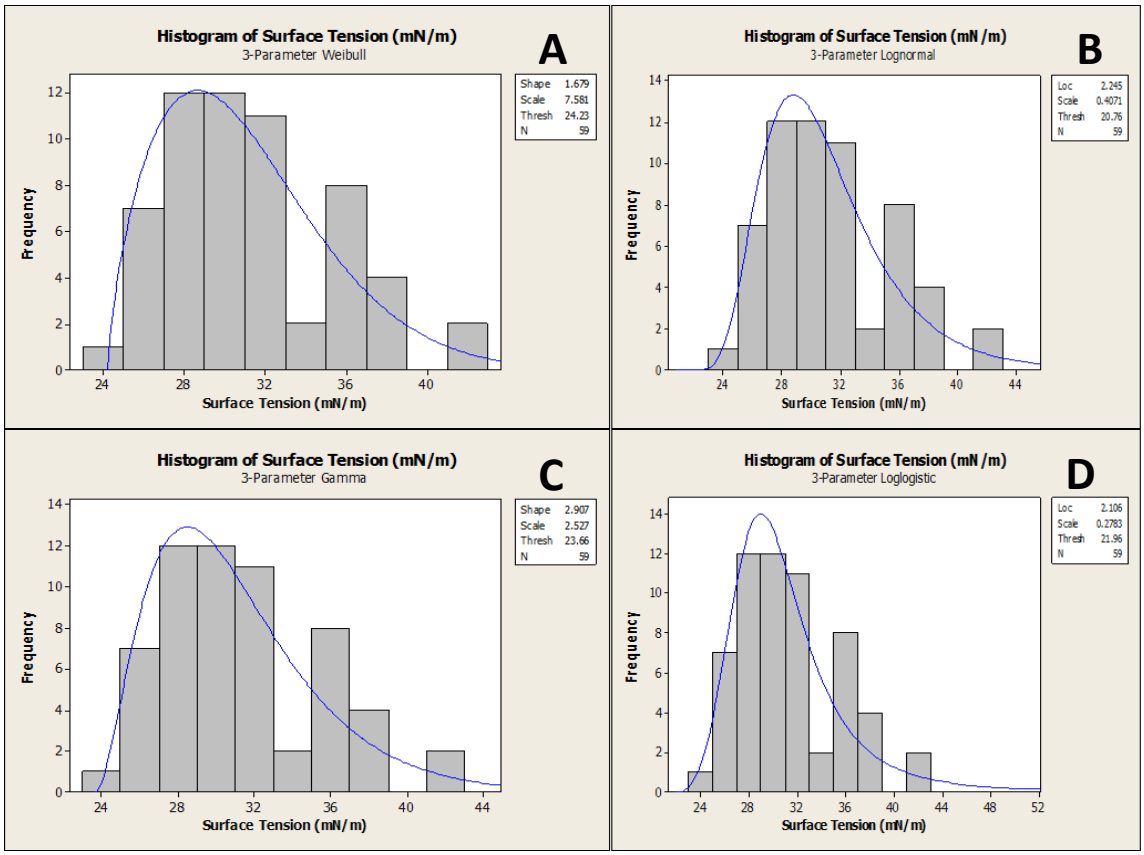


Figure 4.8 The frequency distribution curves predicting the minimum liquid surface tensions of bacteria producing surfactants can achieve. The threshold value of the minimum surface tension values are shown in all the three parameter curves, with A (Weibull, 24.23 $mN.m^{-1}$), B (Lognormal, 20.76 $mN.m^{-1}$), C (Gamma, 23.66 $mN.m^{-1}$) and D (Loglogistic, 21.96 $mN.m^{-1}$). This figure was obtained from SPSS and may not reproduce well.

4.5.3 Comparison of predicted γ_{Min} values determined by IDI analysis and random samples of recently published reports

Individual distribution identification (IDI) analysis based on the Anderson-Darling goodness of fit test was used to identify theoretical probability distributions to fit surface tension data ≤ 42.1 $mN.m^{-1}$, determined in this work for SUDS soil and activated sludge-isolated pseudomonads (Table 4.1) and the surface tension data for parallel set of recently published 59 mixed bacterial spp. (Table 4.3) was also analysed by IDI and this were compared with sets of soil isolated pseudomonads by Fechtner *et al*. (2011). The results of the predicted minimum surface tension (γ_{Min}) of all the three data sets are shown in Table 4.4.

Table 4.4 Predictions from a collection of soil and activated sludge isolated pseudomonads for this work, soil-isolated pseudomonads and from random sample of published reports of mixed bacterial spp.

Data set	Best-fitting 3-parameter distribution*	N	P	AD	γ_{Min} ($mN.m^{-1}$)
Contaminated soil and activated sludge-isolated pseudomonads	Log logistic	50	0.294	0.497	24.24
Soil-isolated pseudomonads (Fetchner *et al*., 2011)	Gamma	38	0.233	0.688	24.16
Random sampling of published reports for mixed bacterial spp.	Weibull & JT	59	0.386	0.238	24.23

Individual distribution identification (IDI) analyses were used to fit theoretical probability distributions to surface tension data, and from the threshold parameters to predict γ_{Min}. N, Number of bacterial isolates; P, *P* value; AD, Anderson-Darling test statistic; *, For all data sets, gamma, log-normal and log-logistic distributions fitted the surface tension data well; additionally, the Weibull distribution and the Johnson transformation of a normal distribution fitted the Random sampling of published reports data well. Only those with surface tensions ≤ 42.1 $mN.m^{-1}$ were used in this analysis, following the upper limit of LSTRA determined here for the contaminated soil and activated sludge–isolated pseudomonads.

4.5.4 Concluding statement

The minimum surface tension (γ_{Min}) of a collection of pseudomonads and *Pseudomonas*-like bacteria from wastewater and SUDS soil was predicted to be close to 24.24 $mN.m^{-1}$. This compares well with predictions made from an earlier collection of soil pseudomonads (Fechtner *et al*. 2011) and from a random sample of published reports of bacterial LSTRA isolates.

4.6 Discussion

Bacteria have been isolated from different environment and the lowest surface tension (γ) produced from small collections of pseudomonads isolated from soil and from water and wastewater environments is in the range of 24 - 35 $mN.m^{-1}$ (as reported by De Bruijn *et al.,* 2007; Perneel *et al.,* 2007; Viramontes-Ramos *et al.,* 2010; Fechtner *et al.,* 2011). In the same vein, the soil bacterium *Bacillus subtilis* reduces γ to between 26 to 27 $mN.m^{-1}$ (Nitschke *et al.,* 2004; Viramontes-Ramos *et al.,* 2010).

A total of 85 pseudomonad isolates, comprising of 71 drop-collapse positive and 14 randomly selected negative isolates were investigated by quantitative tensiometry of cell free culture supernatant to confirm the likely expression of surfactants. This is similar to the method adopted by Youssef *et al.* (2004) in which strains that showed positive or negative results from blood agar, oil spread and drop collapse tests were selected for further quantitative tensiometry .Of these, 57 significantly reduced the surface tension (LSTRA

isolates #69 – #75) and the remaining 28 isolates (non-LSTRA isolates #114 - #311) did not. The LSTRA isolates were further differentiated into the low-γ groups (isolates #69 – #183) and intermediate-γ groups (isolates #5 - #75) respectively. This method differentiates the pseudomonads into different classes of biosurfactant activity by statistical means relative to the control used (Fechtner *et al*., 2011). It is more powerful than the scoring method used by Yessouf *et al.,* (2004) to determine different magnitudes of biosurfactant activity using blood agar, oil spreading and drop collapse tests in which activity was graded from a partial (+) to complete activity (+++) for each of the tests conducted. The value might uncover differences in behaviour but this approach plus quantitative measurements reports rigorous statistical analysis is used to determine the strength of the surfactants produced.

The increase in incubation period was also found to have little impact on the measured surface tension in cultures (Table 4.1), though significant growth increase between the 24 h and 48 h cultures was confirmed by OD_{600} measurements (*t*-test, $P < 0.0001$). It was observed that isolates from the non-LSTRA group tested (#51 and #307), did not show significant change in surface tension after 48 h (56.6 ± 0.3 to 55.6 ± 0.4 $mN.m^{-1}$ and 55.0 ± 0.8 to 59.1 ± 0.5 $mN.m^{-1}$) as their measured surface tensions increases compared to KB control, and this might be due to culture aging or cell lysis as previously reported by Fechtner *et al.* (2011). Three of the Isolates from the intermediate group (#42 #167 and #228) were probably expressing weak surface tension at high levels (36.5 ± 1.0 to 37.5 ± 0.9 $mN.m^{-1}$, 35.0 ± 1.4 to 39.1 ± 1.1 $mN.m^{-1}$ and 38.8 ± 0.7 to 40.3 ± 0.5 $mN.m^{-1}$). Only Isolates #81 and #85 showed significant but minor

decreases in surface tension of 31.9 ± 1.5 to 26.6 ± 0.1 $mN.m^{-1}$ and 28.9 ± 0.1 to 26.9 ± 0.1 $mN.m^{-1}$ ($P \leq 0.05$), which probably meant that both were expressing strong surfactants at low levels. Increased surface tension was also noticed in isolate #303 (25.1 ± 0.0 to 25.4 ± 0.1 $mN.m^{-1}$) which was suspected to be due to culture aging and cell lysis (Fechtner *et al.,* 2011).

The surface tension of individual cultures of isolates and their mixtures chosen from low, intermediate and non-LSTRA groups were investigated to assess the presence of antagonistic compounds. From the results of these mixing experiments, it was observed that there was no significant difference between the surface tension of individual isolates and their mixtures (Fig. 4.2).

In order to predict the minimum surface tension (γ_{Min}) reducing activities of these isolates, analysis of the surface tension data for 50 LSTRA isolates that formed a single large homogeneous group with mean surface tension ≤ 42.1$mN.m^{-1}$ (Fig 4.3) was undertaken using Individual distribution identification (IDI) analysis and results showed that the log-logistic distributions fitted the surface tension data well based on Anderson-Darling goodness of fit (AD = 0.497, $P > 0.293$) predicting γ_{Min} of 24.24 $mN.m^{-1}$ as shown in Fig. 4.4 and the frequency distributions in Fig. 4.5. The three parameter frequency distribution plots obtained from the analysis also supported to the fact that the true γ_{Min} for this collection of pseudomonad isolates is close to 24 $mN.m^{-1}$.

Analysis of the selected 59 mixed bacterial spp. with mean surface tension data ≤ 42.1$mN.m^{-1}$ (Fig. 4.6) as informed by the homogenous grouping table, from a range of bacteria including non-pseudomonads using Individual

distribution identification (IDI) analysis and its frequency distribution (Fig. 4.7 & 4.8), in which the γ_{Min} threshold value of 24.23 mN.m^{-1} was estimated, which also confirmed the findings of this current study.

Finally, the three predictions of the minimum surface tension i.e. from a collection of soil and activated sludge pseudomonads for this work, soil-isolated pseudomonads (Fechtner *et al*. 2011) and from random sample of published reports of mixed bacterial spp. including non-pseudomonads were compared and found to be 24 mN.m^{-1}.

4.7 Conclusion

Surfactant producing isolates were successfully isolated and analysis of the surface tension data showed that the minimum LSTRA achieved for the collection of soil and wastewater bacteria was found to be close to 24 mN.m^{-1}. It should be borne in mind that there appears to be a fundamental limit to the reduction of liquid surface tension of bacterial cultures of 24 mN.m^{-1}, suggesting that additional effort in looking for more active agents will become progressively less rewarding.

Chapter 5

Diversity in Surfactant Behaviour

5.1 Introduction

Surfactants show different behaviours in water where they affect the stability of foams, and in oil-water mixtures where they affect the production and stability of films and emulsions (Williams, 1991; Viramontes-Ramos *et al.,* 2010; Lesov *et al*., 2014; Worthen *et al*., 2014). Surfactant behaviour is highly sensitive to the chemical structure of the surfactant, forming micelles in aqueous phase, where the hydrophobic tails face inwards and the hydrophilic heads are in contact with the surrounding medium. The tails consists of long-chain fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxyl fatty acids, while the head is composed of carbohydrates, amino acids, cyclic peptides, phosphates, carboxylic acids or alcohol groups (Ron, 2001; Wang *et al.,* 2007). The type of structures surfactants form in solution (micelles, bilayers and vesicles) depends on the relative size of the hydrophilic head and the hydrophobic tail (the hydrophilic-lipophilic balance, HLB; Griffin, 1949) and the behaviour of surfactants may or may not be masked or modified by the presence of other compounds, including cells (Batista *et al*., 2006; Koza, 2011).

Surfactants show significant variation in their ability to stabilise foams, oil films and form emulsions and are also able to destabilise these mixtures (Vikingstad *et al.,* 2005; Viramontes-Ramos *et al.,* 2010). These variations in behaviours mean that surfactants can be differentiated without the need to first purify them and establish their chemical structure, and potentially may be suited to different biotechnological applications.

5.2 Used Lubricating Oil (ULO)

Used lubricating oil (ULO) was chosen to test for surfactant behaviour because it is a good model (complex) hydrocarbon with wide range of molecular sizes, mostly comprising aromatic hydrocarbons and some compounds in smaller quantity e.g oxygen, nitrogen and sulfur (Fetter, 1993). The ULO is regarded as a daily used product, with broad circulation and great contamination potential. The presence of different types of automobiles and mechinery has resulted in the use of lubricating oil and the spillage of this product frequently contaminate our air, soil and water and this has drawn the attention of the public because of their toxicity, mutagenic as well as carcinogenic properties (Abioye *et al.,* 2012). The ULO considered in this work is classified as the light crude oil, in that it has low density and flows freely at room temperature.

Many researchers have used diesel, mineral oil to study the rate of degradation of these hydrocarbons. The bioremediation of soil contaminated with 5% and 15% ULO amended with different substrate was studied under laboratory conditions and the highest degradation rate of 92% was recorded after a period

of 84 days on soils with 5% ULO amended with brewery spent grain (Abioye *et al.,* 2012). Diesel oil was used as a source of isolation of certain bacterial strains including *Bacillus subtilis* and *Bacillus cereus*, and found to have hydrocarbon degrading capability (Nwaogu *et al.,* 2008). In another study, to test a series of microbial association that could degrade various petroleum oils, emulsols, and crude oil. An efficient degradation was achieved during aerobic flow cultivation and the oils were degraded to about 92% with the aid of parameters such as brand of oil, the concentration of the oil and the degree of emulsification (Aluyor & Ori-jesu, 2009).

5.3 Research Objectives

The aim of the research reported in this chapter was to investigate whether the surfactants expressed by a set of low-γ LSTRA (liquid surface tension reducing activity) pseudomonad isolates exhibited variations in water and oil-water behaviour that might suggest that the surfactants themselves had varying chemical structures. This information would be helpful for the selection of isolates for future chemical analyses and testing in bioremediation and biotechnology.

The objectives of this research were to:

1. Determine whether the set of 41 low-γ LSTRA isolates represents a diverse collection of strains using the phenotype data;

2. Determine the water and oil-water behaviours of surfactants produced by the low-γ LSTRA isolates, using foam stability, emulsion and oil-displacement assays;

3. Identify a sub-set of isolates suitable for future chemical analysis and testing using correlation and clustering analysis of behavioural data; and

4. Investigate whether there is evidence to support chemical variation within surfactants expressed by sub-sets of isolates producing very similar surface tensions.

5.4 Determining the differences in phenotypic expressions of the 41 low-γ LSTRA isolates

In order to determine whether the 41 low-γ LSTRA isolates identified in the previous Chapter 4 (Section 4.3.1, Page 63) from a larger collection of 85 strains investigated for surfactant expression still represents a diverse collection of strains, hierarchical cluster analysis of the phenotype data obtained for these strains (Chapter 3, Section 3.4.3, Page 54) was undertaken and the dendrogram shown in Figure 5.1 (Page 91). The 41 isolates could be grouped into 37 clusters of between 1 - 2 isolates based on phenotype similarity using the natural break in the corresponding scree plot. This analysis demonstrates that this collection of low-γ LSTRA pseudomonads still represents a diverse group of isolates with a minimal level of biological replication, and likely therefore to show variation in the surfactants they express.

5.5 Determining surfactant behaviours by foaming, emulsion and oil-displacement assays

The behaviour of surfactant solutions produced by low-γ LSTRA pseudomonad isolates can be described by investigating emulsion-formation produced by mixing oil and water, the extent surfactant solutions can displace a thin layer of oil on water surfaces, and how surfactants stabilised foams.

5.5.1 Foam stabilisation

The behaviour of surfactants produced by low-γ LSTRA pseudomonad isolates was investigated by the stabilisation of foams after the method of Sathe and Salunke (1981). In these assays, replicate 18 h KB cultures were shaken to produce foam, with foam stability measured as the foam reduction height (%) after 2 h. Mean foam stability of the pseudomonad cultures are shown in Figure 5.2 where significant differences were observed between isolates (ANOVA, $P < 0.001$).

Although most foam were stable, suggesting that the surfactants had little impact on foam reduction, one isolate (#88) showed substantial instability with the foam completely drained over the 2 h period of the assay. These observations of foam stability suggest that there is chemical variation in the surfactant each low-γ LSTRA pseudomonad expresses under the conditions used here.

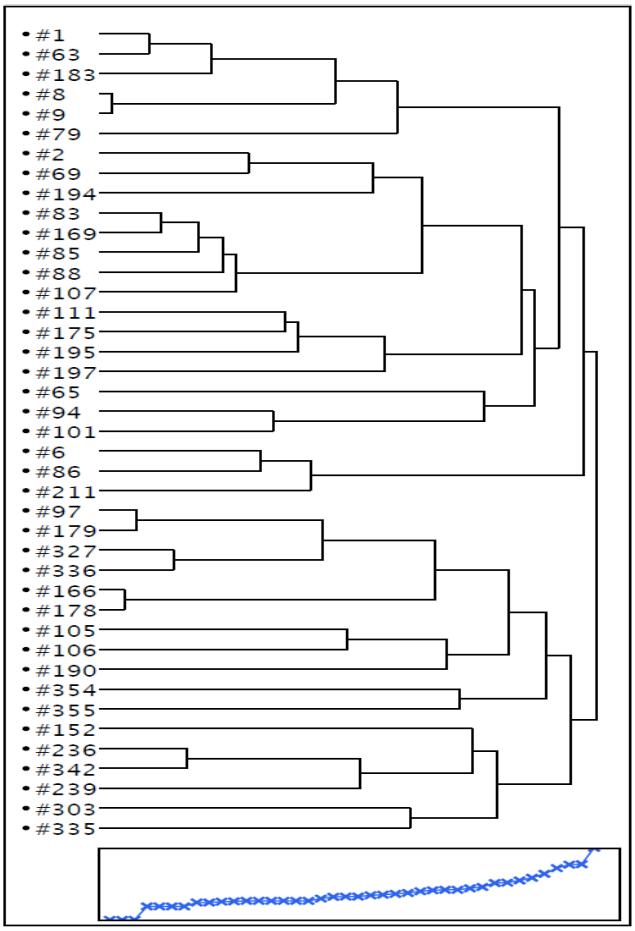


Figure 5.1 The low-γ LSTRA pseudomonad isolates show differences in phenotype. Hierarchical cluster analysis of phenotype data for the 41 low-γ LSTRA pseudomonad isolates groups them into at least 37 clusters of 1 – 2 isolates based on phenotype similarity and the natural break in the corresponding scree plot. Isolates linked by deeper branches (e.g. #1 and #79) are more dissimilar than isolates linked by shallow branches (e.g. #1 and #63). Isolates are indicted by their strain numbers, #1 – #335. This dendrogram links isolates on the basis of similarity, and is shown with an even-spacing horizontal scale and a scree plot at the bottom where the ordinate shows the distance that was bridged to join the clusters at each step.

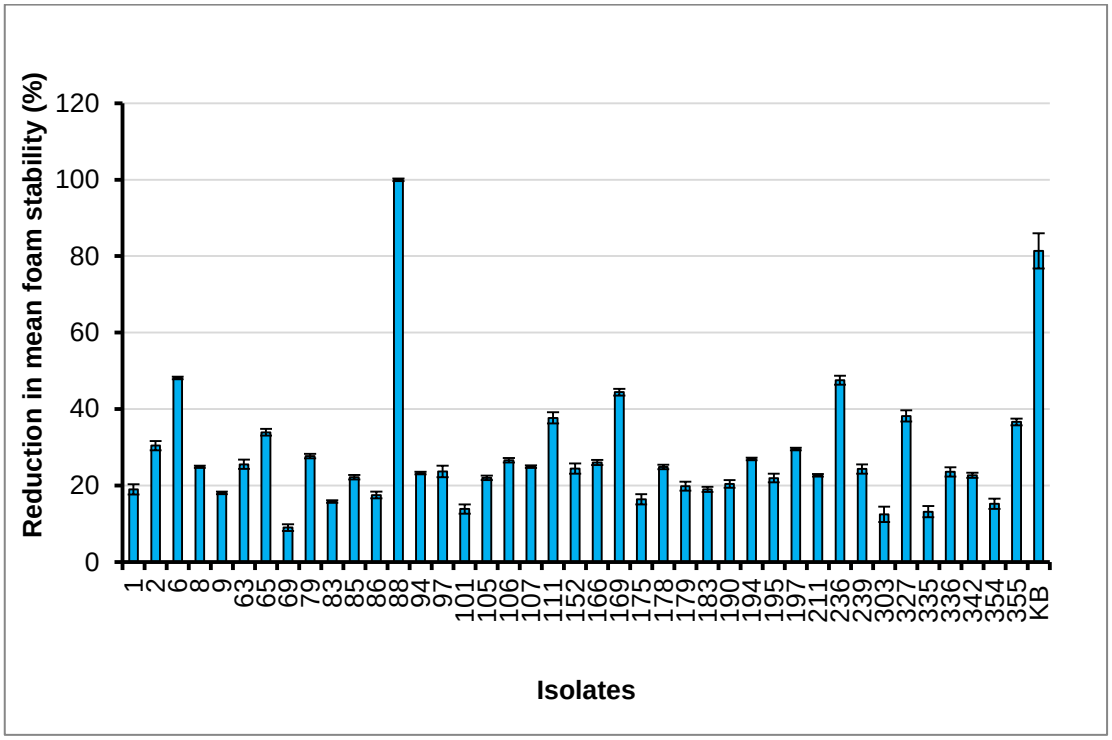


Figure 5.2 Effect of surfactants expressed by the low-γ LSTRA pseudomonad isolates on reduction in mean foam stability. Foam stability assays were carried out using replicate 18 h KB cultures (n = 3) with mean ± SE of reduction in foam stability (%) over 2 h shown. A significant difference between isolates was observed (ANOVA, P < 0.001). Isolates are indicted along the x-axis by their strain numbers, #1 – #355, and a negative control was provided by sterile KB samples.

5.5.2 Emulsion and oil displacement assays

The behaviour of surfactants produced by low-γ LSTRA pseudomonad isolates was investigated by the production of oil emulsions and by oil-displacement assays. Emulsions were produced using used lubricating oil (ULO) and 18 h KB cultures, and the relative oil (O_i), aqueous (A_i), and emulsion (E_i) indices determined.

Oil-displacement was determined using ULO and 18 h KB cultures, with the mean oil-displacements shown in Figure 5.6, where a significant difference was observed between isolates (ANOVA, $P < 0.001$). These observations of emulsion and oil-displacement behaviour suggest that there is chemical variation in the surfactant each low-γ LSTRA pseudomonad expresses under the conditions used here. This suggestion is further supported by the phenotyping and analysis of the isolates (Chapter 3) which indicated that the collection of pseudomonads was likely to contain a low level of biological replication (i.e most isolates could be shown to be different from all others). The apparent diversity in isolate phynotype could therefore explain the diversity in surfactants expressed.

A low oil index is the result of the surfactant moving more oil into the emulsion phase, a low emulsion index (E_i) is the result of the surfactant moving more oil into the emulsion phase, and a low aqueous index (A_i) is the result of the surfactant moving more liquid into the emulsion phase. Mean indices for the pseudomonad cultures are shown in Figures 5.3 (Page 95), 5.4 (Page 96), and

5.5 (Page 97), where significant differences were observed between isolates (ANOVA, $P < 0.001$).

5.5.3 Concluding statement

An investigation of the oil-water behaviour of surfactants expressed by foaming, emulsion and oil-displacement assays has demonstrated that there were significant behavioural differences ($P < 0.001$) within this set of 41 low-γ LSTRA pseudomonad isolates. This suggests that these isolates express surfactants with differing chemical structures, and differing hydrophilic-lipophilic balances might therefore explain the ability of some isolates to better stabilize foams or emulsions than others.

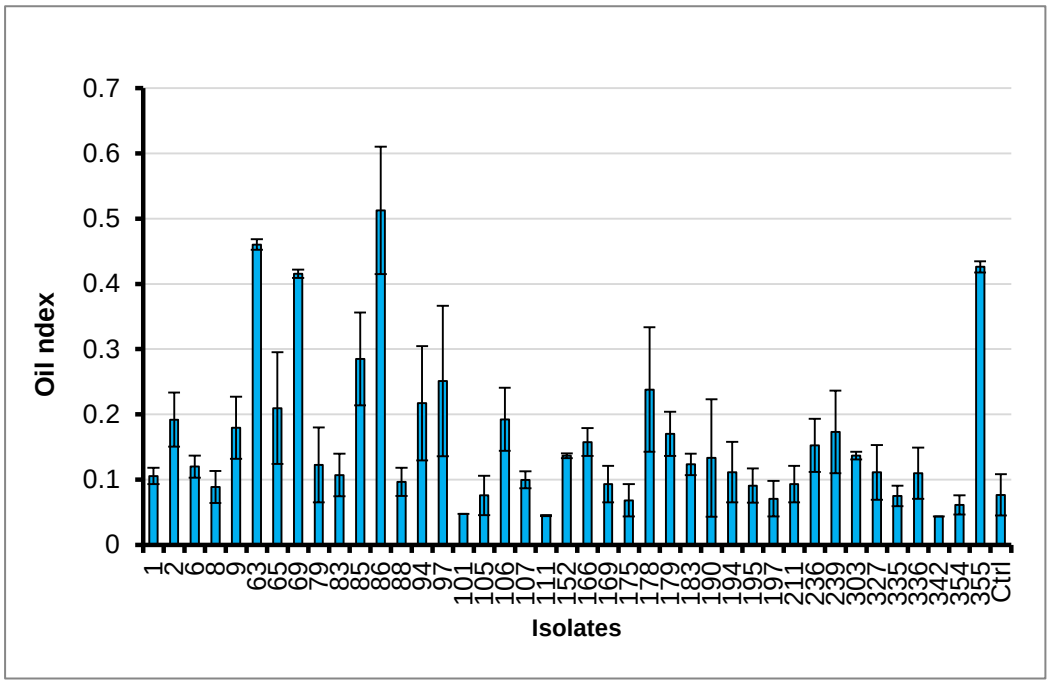


Figure 5.3 Effect of surfactants expressed by the low-γ LSTRA pseudomonad isolates on emulsification and the oil index (O_i). Emulsion assays were carried out using replicate 18 h KB cultures, deionised water and used lubrication oil (ULO) (*n* = 3) with the mean ± SE oil index (O_i) shown. A significant difference between isolates was observed (ANOVA, *P* < 0.001). Isolates are indicted along the x-axis by their strain numbers, #1 – #355 and a negative control was provided by an oil, deionised water and water mixture.

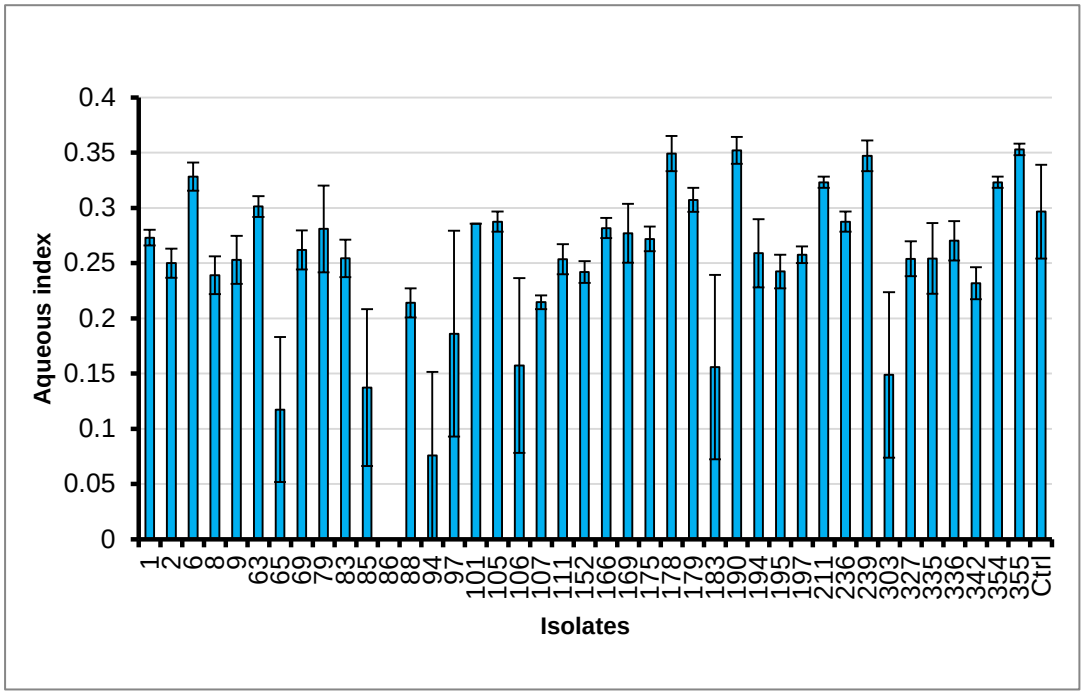


Figure 5.4 Effect of surfactants expressed by the low-γ LSTRA pseudomonad isolates on emulsification and the aqueous index (A_i). Emulsion assays were carried out using replicate 18 h KB cultures, deionised water and used lubrication oil (ULO) (n = 3) with the mean ± SE aqueous index (A_i) shown. A significant difference between isolates was observed (ANOVA, $P < 0.001$). Isolates are indicted along the x-axis by their strain numbers, #1 – #355 and a negative control was provided by an oil, deionised water and water mixture.

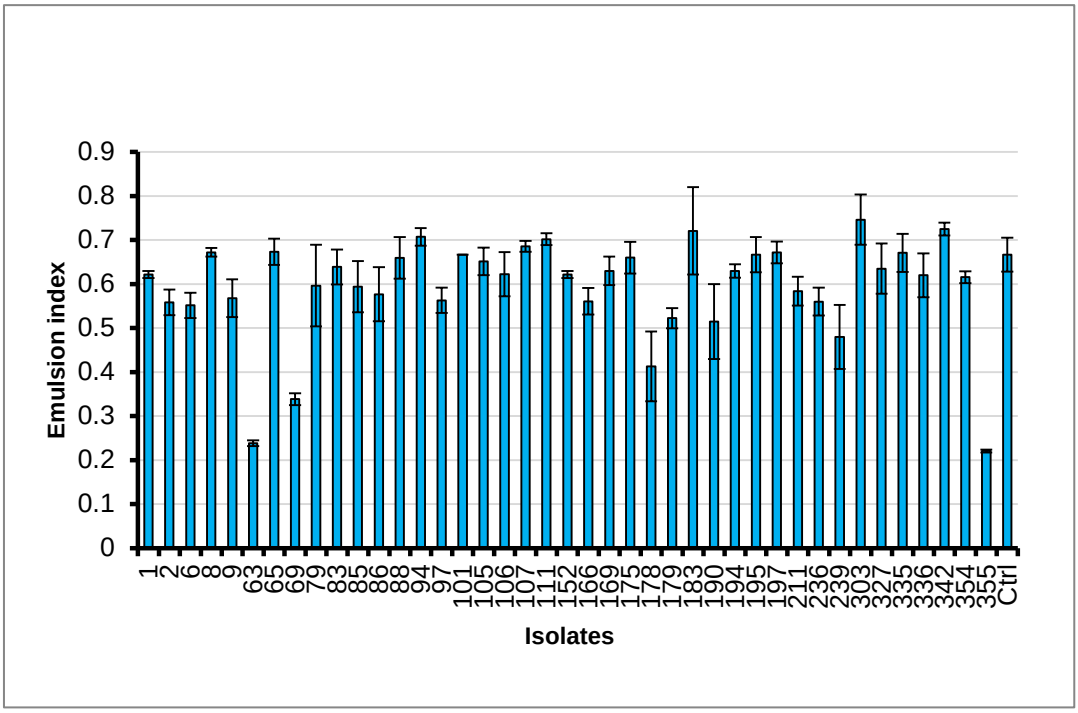


Figure 5.5 Effect of surfactants expressed by the low-γ LSTRA pseudomonad isolates on emulsification and the emulsion index (E_i). Emulsion assays were carried out using replicate 18 h KB cultures, deionised water and used lubrication oil (ULO) (n = 3) with the mean ± SE emulsion index (E_i) shown. A significant difference between isolates was observed (ANOVA, $P < 0.001$). Isolates are indicated along the x-axis by their strain numbers, #1 – #355 and a negative control was provided by an oil, deionised water and water mixture.

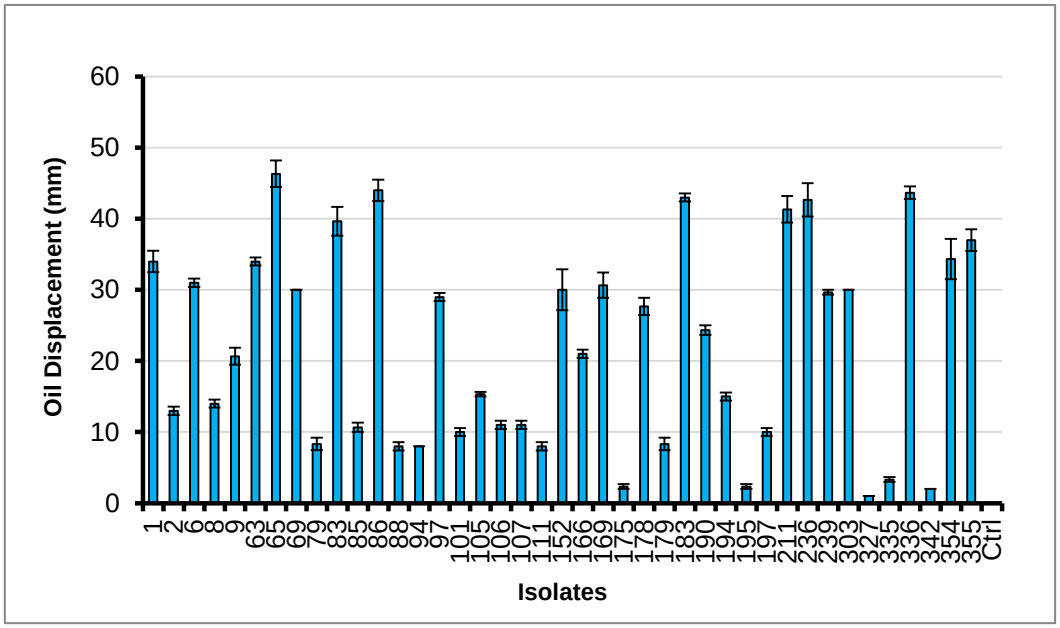


Figure 5.6 Effect of surfactants expressed by the low-γ LSTRA pseudomonad isolates on oil displacement activity. Oil displacement assay was carried out using replicate 18h KB cultures (*n* = 3) with mean ± SE oil displacement shown. Significant differences between isolates were observed (ANOVA, *P* < 0.001). Strains are indicated along the x-axis by their strain numbers, #1 – #355 and a negative control was provided by sterile water and oil.

5.6 Investigating surfactant behaviour to uncover evidence of structural diversity

In order to identify a sub-set of isolates from the larger set of 41 low-γ LSTRA pseudomonad isolates expressing potentially-novel surfactants for future chemical analysis and testing, the foam stability, emulsion and oil-displacement data obtained in Section 5.5 (Page 90) were evaluated using pairwise correlations and hierarchical cluster analysis.

5.6.1 Pairwise correlations and Hierarchical cluster analysis

In order to assess whether any one particular isolate expressed a surfactant with unusual properties as determined by the surfactant behaviour assays (Section 5.5, Page 90), all pairwise correlations were determined, with 5 of the 15 correlations found to be significant ($P \leq 0.05$) (Table 5.1, Page 100). The scatter plots of each of the significant correlations (emulsion index (E_i) vs oil-displacement, oil index (O_i) vs oil-displacement, aqueous index (Ai) vs emulsion index (E_i), and emulsion index (E_i) vs oil index (O_i); Appendix A3) were then inspected visually to identify isolates with 'unusual' behaviours (i.e. outliers) which are listed in Table 5.2 (Page 101). This identified 21 strains (51% of the total), including a number which were identified multiple times. Although this approach is semi-qualitative, this approach could be used to identify isolates to be targeted in future investigations of surfactant structure and behaviour. For example, if sufficient resources were available to examine two strains only,

Isolates #63 and #69 should be tested; however, if five could be examined, isolates #63, #69, #86, #342 and #355 should be tested.

Table 5.1 Pairwise correlations of surfactant behaviour

Factor	Factor	R^2	*P*
Aqueous index	Emulsion index	0.204	0.003
Foam reduction	Surface tension	0.002	0.790
Foam reduction	Emulsion index	0.000	0.947
Foam reduction	Aqueous index	0.003	0.740
Foam reduction	Oil index	0.011	0.512
Oil displacement	Aqueous index	0.000	0.986
Oil displacement	Emulsion index	0.114	0.031
Oil displacement	Foam index	0.006	0.636
Oil displacement	Oil index	0.150	0.013
Oil displacement	Surface tension	0.085	0.064
Oil index	Aqueous index	0.074	0.086
Oil index	Emulsion index	0.532	0.000
Surface tension	Aqueous index	0.034	0.252
Surface tension	Emulsion index	0.013	0.476
Surface tension	Oil index	0.001	0.879

Oil (O_i), aqueous (A_i) and emulsion (E_i) indices were determined as the relative height of each layer after shaking a mixture of 18 h KB culture and oil after 24 h (*n* = 3). Foam stability was determined as the percentage reduction of foam heights after 2 h using 18 h KB cultures (*n* = 3). The displacement of an oil film by a drop of 18 h KB culture was measured as the drop diameter (mm) after 5 s (*n* = 3). Significant correlations were observed between A_i *x* E_i (R^2 = -0.4510, *P* = 0.0031), O_i *x* E_i (R^2 = -0.7295, *P* < 0.0001), Oil-displacement *x* E_i (R^2 = -0.3368, *P* = 0.0313) and Oil-displacement *x* O_i (R^2 = 0.3867, *P* = 0.0125); all other pair-wise correlations were not significant (*P* ≤ 0.05).

An alternative approach using Hierarchical cluster analysis (HCA) was also used in an attempt to identify isolates for future investigation (although the correlation-based analysis and HCA are not strictly independent as they both use the same data set). A dendrogram was produced using the surfactant

behaviour data in which isolates were grouped together based on similarity and is shown in Figure 5.7 (Page 102).

This analysis provides evidence of diversity in the surfactant behaviour expressed by this set of 41 low-γ LSTRA pseudomonad isolates, and can be used to select isolates for further analysis by identifying pairs of isolates that are grouped by deep branches (i.e. are most dissimilar). For example, if sufficient resources were available to examine two strains only, isolates #69 and #342 should be tested; however, if five could be examined, isolates #65, #69, #86, #169 and #342 should be tested (it is noteworthy that several of the isolates identified by the correlation analysis group together in this cluster diagram, providing further reason why these strains should be targeted first).

Table 5.2 Identification of isolates for future investigation as informed by the inspection of scatter plots of surfactant behaviours

	Groups of isolates[a]				
Correlations	**1**	**2**	**3**	**N[b]**	**(%)[c]**
Emulsion index (E_i) and Oil displacement	#342	#63, #69, #178 & #355	-	5	12.2
Oil index (O_i) and Oil displacement	#63, #69, #86 & #355	#83, #175, #195, #327, #335 & #342	#65, 185, #211, #236 & #336	15	36.6
Aqueous index (A_i) and Emulsion index (E_i)	#63, #69 & #355	#65, #94, #183 & #303	#86	8	19.5
Emulsion index(E_i) and Oil index(O_i)	#101, #111& #342	#63, #69 & #355	#86	7	17.1

Summary of an inspection of individual scatter plots where a maximum of three groups of strains showing 'extreme' behaviour were identified; 'a' are the arbitrary groups; 'b' is the total number of isolates identified; and 'c' is the percentage of the total isolates identified in this manner

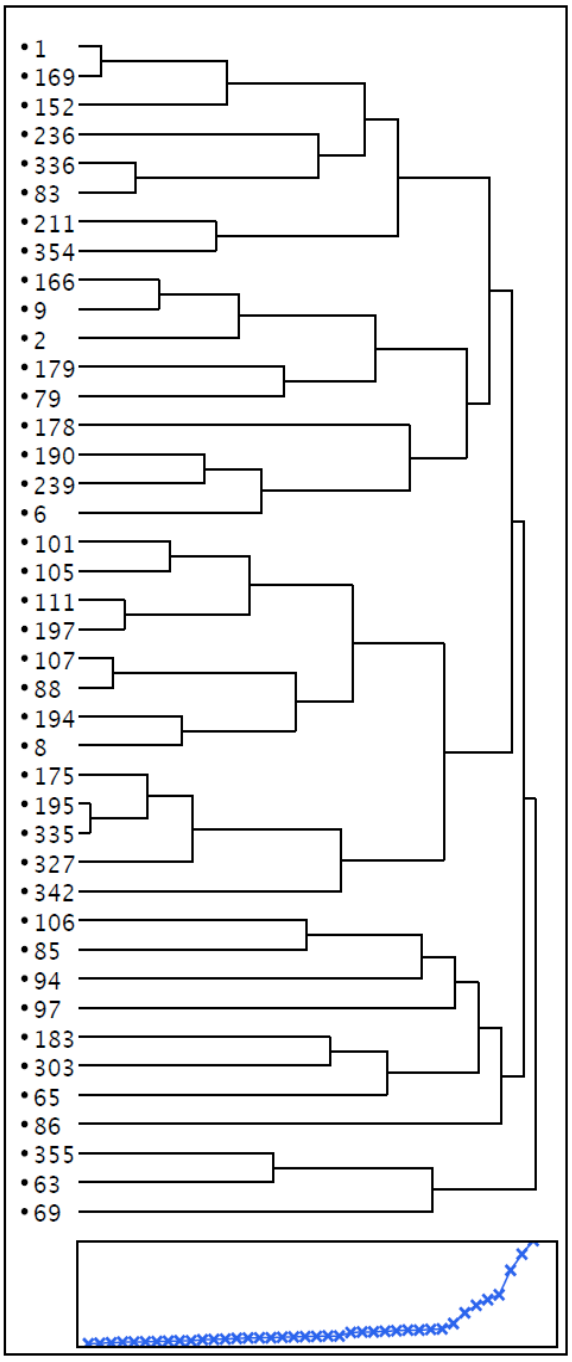


Figure 5.7 The low-γ LSTRA pseudomonad isolates express a diverse collection of surfactant types. Hierarchical cluster analysis of surfactant behaviour data for the 41 low-γ LSTRA pseudomonad isolates groups them into at least 10 clusters of 1 – 8 isolates based on similarity and the natural break in the scree plot, suggesting that more closely-related isolates will produce similar surfactant types than more distantly related isolates. Isolates are indicted by their strain numbers. This dendrogram links isolates on the basis of similarity, and is shown with an even-spacing horizontal scale and a scree plot at the bottom where the ordinate shows the distance that was bridged to join the clusters at each step.

5.6.2 Concluding statement

This analysis of the surfactant behaviour data obtained for the low-γ LSTRA isolates using pairwise correlations and Hierarchical cluster analysis provides two different means to identifying strains for future investigation of potentially novel surfactants (see Table 5.2 and Fig 5.7, Pages 101 and 102, respectively). Isolates that were identified in the correlation analysis could also be considered using the HCA dendrogram based on diversity of surfactant behaviours to further refine a selection of strains for future analyses. For example, if sufficient resources were available to examine two strains only, then we propose that isolates #69, #342 should be considered, and if four isolates are to be examined, then isolates #69, #86, #169 and #342, should be considered.

5.7 Investigating low-γ LSTRA isolates producing cultures with small differences in surface tension

In order to investigate whether there was evidence to support variation in surfactant type expressed by sub-sets of low-γ LSTRA isolates producing very similar surface tensions, the first 30 low-γ LSTRA isolates shown in Chapter 4 (Section 4.3.1, Figure 4.1, Page 65-66 were examined by a post hoc comparison of means. These analyses identified two homogeneous groups of isolates (Table 5.3, Page 102) producing significant, but small differences in surface tensions (TK-HSD, $\alpha = 0.05$; Group I: 25.2 ± 0.1 mN m^{-1} and Group II: 26.7 ± 0.5 mN m^{-1}).

Only 27 isolates could be grouped using this method, and the remaining 3 isolates falling between the Group I and II were discarded (additional strains with decreasing LSTRA were tested in a similar manner, but did not add to Group II and were not considered further). The ability to differentiate these 27 isolates into two distinct groups by a statistical comparison of surface tensions provides some evidence to suggest that there is some variation in surfactant types expressed by these strains. These two groups of isolates were further analysed using hierarchical cluster analyses (HCA) in the next section.

Table 5.3 List of isolates that formed the homogeneous group and their mean surface tension

Isolates	Group	Liquid surface tension ($mN.m^{-1}$)
#83	I	24.8 ± 0.1
#101	I	24.9 ± 0.0
#303	I	25.1 ± 0.0
#190	I	25.1 ± 0.1
#152	I	25.1 ± 0.0
#178	I	25.2 ± 0.4
#6	I	25.2 ± 0.2
#1	I	25.3 ± 0.1
#65	I	25.3 ± 0.2
#211	I	25.3 ± 0.1
#194	I	25.4 ± 0.1
#79	I	25.5 ± 0.1
#179	I	25.5 ± 0.1
#86	II	25.5 ± 0.1
#236	II	26.4 ± 0.1
#197	II	26.4 ± 0.0
#169	II	26.4 ± 0.1
#88	II	26.5 ± 0.1
#239	II	26.6 ± 0.1
#175	II	26.7 ± 0.2
#166	II	26.7 ± 0.2
#335	II	26.7 ± 0.1
#336	II	26.7 ± 0.1
#355	II	26.8 ± 0.1
#105	II	26.8 ± 0.1
#107	II	26.9 ± 0.1
#111	II	26.9 ± 0.2

The liquid surface tension of 27 isolates that formed the homogeneous groups, listed in increasing surface tension order. Means and standard errors are shown. Liquid surface tensions were determined by tensiometry of cell-free 18 h KB cultures (n = 4).

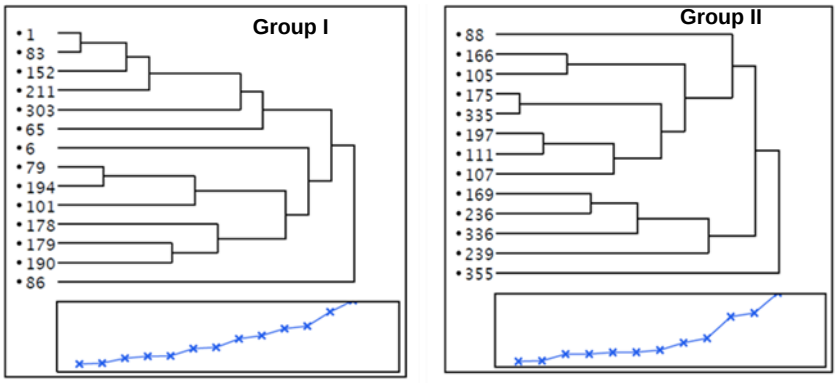


Figure 5.8 The surfactant behaviour data suggests variation in surfactant types expressed within each of the two groups of low-γ LSTRA isolates Hierarchical cluster analysis of the surfactant behaviour data (i.e oil-displacement and emulsion assays) of two groups of isolates produced these clusters (group A, into at least 4 clusters and group B into 5 clusters based on similarity). Isolates linked by deeper branches are more dissimilar than isolates linked by shallow branches. Isolates are indicted by their strain numbers, #1 – #86 and #88 – #355. This dendrogram links isolates on the basis of similarity, and is shown with an even-spacing horizontal scale and a scree plot at the bottom where the ordinate shows the distance that was bridged to join the clusters at each step.

In order to determine further evidence for variations in surfactant type expressed by the two groups of low-γ LSTRA isolates, a hierarchical approach was used to cluster each group of isolates using the surfactant behaviour (Figure 5.8, Page 105). These analyses successfully divided the Group I isolates into at least 4 clusters of 1 – 6 isolates, and Group II isolates into 5 clusters of 1 – 7 isolates based on similarity of surfactant behaviour. This demonstrates that there is variation in surfactant type expressed by the two groups of low-γ LSTRA isolates.

Evidence for variation in surfactant type amongst the low-γ LSTRA isolates was found by a *post hoc* comparison of means of surface tension data which could clearly divide most of the isolates into two homogeneous groups (Group I and II). An analysis of the surfactant behaviour data for each of these groups provided further evidence of variations in surfactant type expressed by these isolates.

5.8 Comparison tests to check for dependence amongst isolates expressing homogeneous surfactants.

The significant differences in surface tension and in surfactant behaviour observed in the low-γ LSTRA isolates (Section 5.6) might be explained by two groups of related isolates; one expressing a particular set of surfactants that result in a lower surface tension than a slightly different set of surfactants produced by the second group. In order to test this possibility, significant

associations (or independence) between surface tension and phenotype, and between surface tension and surfactant behaviour, were assessed using a 2 *x* 2 contingency table approach. This analyses required the two groups to be sub-divided into two subgroups (Phenotypes: Subgroups A and B; and Surfactant Behaviours, Subgroups 1 and 2) based on similarity using hierarchical cluster analyses of the phenotype and surfactant behaviour data, respectively (Figure 5.9, Page 108-109). The sorting of Group I and II isolates into each of Subgroups A and B and in Subgroups 1 and 2 ('Outcomes') then provides the data for contingency analyses (Tables 5.4 and 5.5, Page 107).

Table 5.4 Contingency table analysis of surface tension and phenotype.

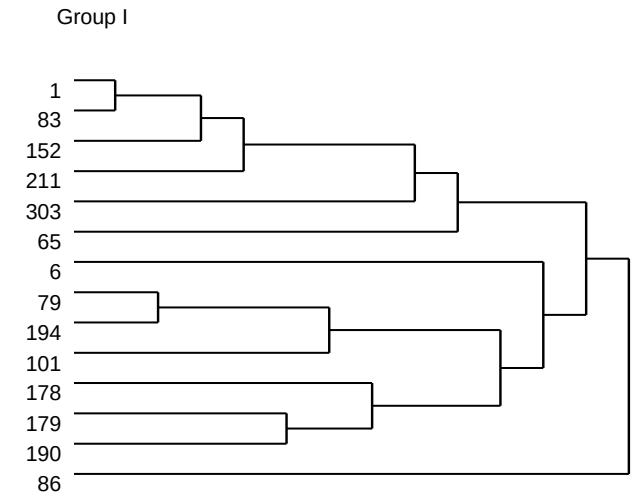
	Phenotype		
Group	**Subgroup A**	**Subgroup B**	**Total**
Group I	8	6	14
Group II	12	2	14
Total	20	8	28

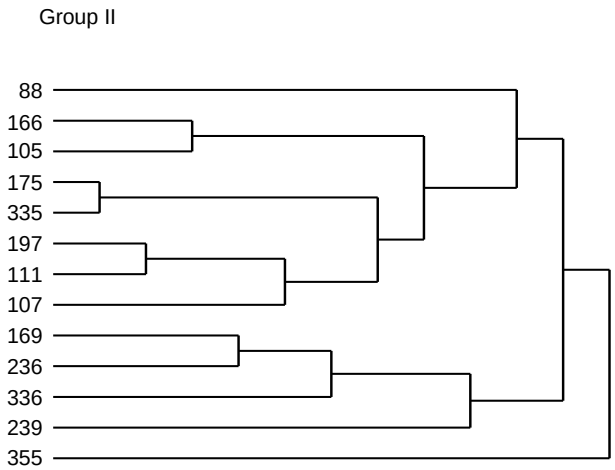
Fisher's Exact Test was used in the analysis of the contingency table and this demonstrates that the homogeneous groups and the HCA grouping patterns are not dependent i.e. statistically not significant (Phenotype, $P = 0.2087$).

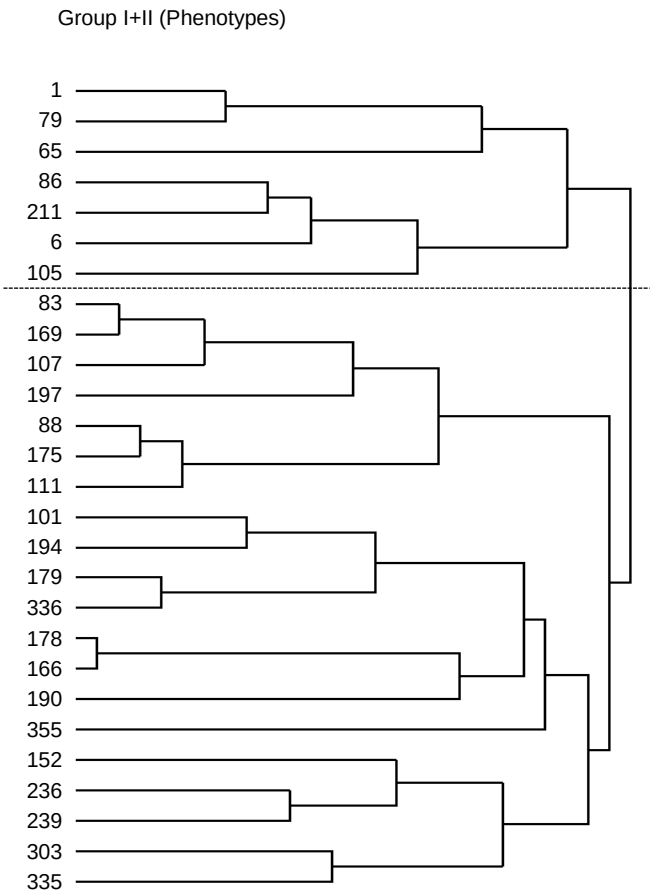
Table 5.5 Contingency table analysis of surface tension and surfactant behaviour.

	Behaviour		
Group	**Subgroup 1**	**Subgroup 2**	**Total**
Group I	3	11	14
Group II	7	7	14
Total	10	18	28

Fisher's Exact Test was used in the analysis of the contingency table and this demonstrates that the homogeneous groups and the HCA grouping patterns are not dependent i.e. statistically not significant (Oil-water behaviour, $P = 0.236$).


Group I
1
83
152
211
303
65
6
79
194
101
178
179
190
86


Group II
88
166
105
175
335
197
111
107
169
236
336
239
355


Group I+II (Phenotypes)
1
79
65
86
211
6
105
83
169
107
197
88
175
111
101
194
179
336
178
166
190
355
152
236
239
303
335

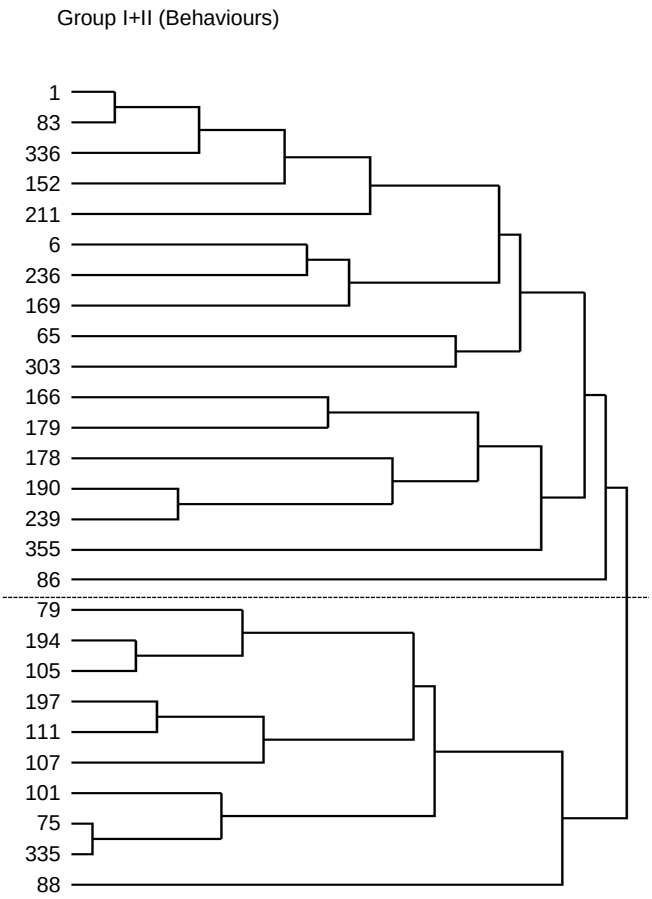

Group I+II (Behaviours)
1
83
336
152
211
6
236
169
65
303
166
179
178
190
239
355
86
79
194
105
197
111
107
101
75
335
88

Figure 5.9 (Previous page)There is little difference between the LSTRA of the top 30 isolates, with evidence to suggest that they differ phenotypically and that the surfactants they produce have different oil-water behaviours. A *post hoc* comparison of the γ data can identify two homogeneous groups of isolates (TK-HSD, α = 0.05) (I & II). Hierarchical cluster analyses (HCA) group these isolates differently using phenotype and oil-water behaviour data. This confirms that there is variation in the type of surfactant they expressed.

Significant levels of association were not observed between the surface tension groups and isolate phenotype (Fisher's Exact Test, P = 0.2087) and surfactant behaviour (P = 0.2365) outcomes. These results indicate that surface tension and isolate phenotype, and surface tension and surfactant behaviours are not dependent, suggesting that within each of the surface tension groups (I and II), the isolates and the surfactants they express are likely to be very different from one another.

These analyses also demonstrated that the low-γ LSTRA isolates are not phenotypically homogeneous, nor do the surfactants they express show homogeneous patterns of oil behaviour.

5.9 Discussion

The overall aim of the work presented in this chapter was to determine whether the surfactants produced by the 41 low-γ LSTRA isolates show diversity using their phenotype and their oil-water behaviour data. We are also interested in identifying a sub-set of isolates for use in future chemical analysis and to find

evidence that support chemical variation within surfactants expressed by these sub-sets of isolates producing very similar surface tension.

The cluster analysis of the 41 LSTRA isolates showed a diverse collection with 1 – 8 isolates grouped into 10 clusters based on phenotype similarity. This showed a diverse group of isolate and therefore influences the type of surfactants they express as shown in Figure 5.1.The scree plots showed some levels of variations amongst the groups and this showed the levels of similarity. It has been reported that the bioremediation activity is mostly influenced by the type of surfactant's chemical structure and other physicochemical conditions of its environmental medium (Reviewed by Ivanković & Hrenović, 2010).

Oil-water behaviour of the low-γ LSTRA pseudomonads isolates clearly demonstrated that there were significant behavioural differences between isolates ($P \leq 0.001$) observed in all the assays conducted under the same conditions (Figures 5.2, 5.3, 5.4, 5.5 and 5.6). These differences in surfactant behaviour amongst the isolates might be due to the nature of chemical structures of the surfactants expressed (Abbasi *et al*., 2012; Karnwal & Bhardway, 2014). In some cases unusual properties or behaviours was noticed (e.g. Isolate #88, Figure 5.2). This showed that these sets of isolates might be exhibiting different chemical variations in relation to the type of structure, the hydrophilic-lipophilic balanced value inherent in the surfactants they produced under the same conditions.

Investigations to uncovering the structural diversity of the surfactants by statistical correlations of oil behaviour showed that 5 of the 15 correlations were

significant. The five significant correlations observed where examined in detail. Inspection of correlation scatter plots identified isolates with similar unusual behaviour are grouped together as shown in Appendix A3 and then selection was based on isolates with the highest number of occurrences (Table 5.2) in all the correlations as the first priority in terms of choice of strains for further analysis.

An alternative method to the aforementioned semi-quantitative approach is the HCA methods in which isolates are grouped on the basis of similarities using the surfactant behaviour data (Figure 5.7). This showed evidence of diversity in behaviour and therefore, the choice of novel isolates were made amongst most dissimilar and a choice between similar groups.

In order to further confirm that variation in behaviour exist among the two homogeneous groups (Group I and II) of isolates producing significantly but small differences in surface tensions (γ = 24 – 27 $mN.m^{-1}$). The hierarchical cluster analysis of the two homogeneous groups of isolates was done and results showed that the isolates exhibit different chemical-structural variations and therefore the surfactants they produce is likely to be different as shown in Figure 5.8.

Significant associations between the homogeneous group, the phenotypes and their behaviour in oils were also tested using a 2 x 2 contingency approach. Some isolates from group I or II are found in either phenotype HCA (A & B) and in the oil behaviour HCA (A & B) as shown in Figure 5.9. This also confirms that they are likely to be very different bacteria having different chemical structures

and the results from the Fisher's Exact Test further confirm that the homogeneous groups, the phenotype and oil-water behaviour are not dependent (Phenotype, P = 0.2087 and Oil-water behaviour, P = 0.2365) as shown in Tables 5.4 and 5.5.

Finally, isolates showed diversity from all the cofirmatory tests by using their phenotype and their oil-water behaviour. These aforementioned differences in surfactant behaviour confirmed through different means in this study can further be supported by Hu *et al.,* (2010) who reported that biological surfactants exhibit some properties which is completely absent in synthetic surfactants, especially in the aspects of biological degradation, safety and physiological activity. For surfactants carrying molecule with amphiphilic structure, the effects of their structures remained a function of their various interfacial behaviour which is directly related to the intermolecular interactions between the surfactant molecules themselves and between surfactant molecules and those present in the system (i.e solvent).

5.10 Conclusion

The work reported in this chapter confirms that this set of 41 low-γ LSTRA isolates identified in Chapter 4 represents a phenotypically diverse collection of isolates. Analysis of the emulsion and oil-displacement behaviour of cultures produced by these isolates suggests that they are expressing surfactants with differing chemical structures. The apparent diversity in the surfactants expressed by these isolates relates to the diversity observed in isolate

phynotypes (Chapter 3). Further evidence of different surfactant types was provided by analyses of the surface tension data of the lowest 30 low-γ LSTRA isolates in which the isolates could be divided into two homogeneous groups. Hierarchical cluster analyses of the phenotype and surfactant behaviour data for each of the two groups showed evidence of variation, and a contingency analysis of these isolates showed that surface tension and phenotype and surfactant behaviour were not associated. These findings all suggest that the low-γ LSTRA isolates are likely to be expressing surfactants with differing chemical structures that can all achieve significantly low surface tensions in cultures.

Chapter 6

Characterization of biofilms produced by *Pseudomonas* spp. in experimental microcosms

6.1 Introduction

Biofilms are assemblages of microorganisms sticking to each other and attached to a surface surrounded by extra-cellular polymeric substance (EPS) (for reviews see O'Toole *et al.,* 2000; Van Colen *et al.,* 2014). Biofilm formation is initiated when free-floating bacteria (planktonic) suddenly change to a sessile-lifestyle to form a surface-attached community based on biotic or abiotic surfaces (see reviews by Moreau-Marquis *et al.,* 2008; Gu & Ren, 2014).

The EPS can be composed of proteins, lipids, polysaccharides, nucleic acids, phospholipids and humic substances (reviewed by Flemming *et al.,* 2013; Liu *et al.,* 2013), of which the major components of the EPS are the proteins and polysaccharides in the range of 70 - 90% in a biofilm (Sheng *et al.,* 2010; Gao *et al.,* 2011).

Bacteria in biofilms develop structures which enable them survive physical, chemical and biological challenges in the environment. This EPS helps protects the biofilm community from physical and chemical attack and against adverse

conditions (reviewed by Abdel-Aziz & Aeron, 2014). Biofilms may also produce surfactants that help to solubilise hydrophobic and other recalcitrant compounds that are not easily accessible (reviewed by Edward & Kjellerup, 2013). This is of great interest particularly when biofilms are to be applied in bioremediation and transformation of noxious and harmful chemicals, particularly if the contaminants are in high concentration that may be harmful to the planktonic microorganisms (reviewed by Edward & Kjellerup, 2013).

It has also been observed from many studies that biofilm bacteria were able to tolerate high concentrations of biocides due to the formation of multi-layered or high cell density structures that hinders or blocks the diffusion of antimicrobials into the centre of the biofilm (Simo˜es & Vieira, 2009; Yuan *et al.,* 2014). The antibiotic concentration needed to kill planktonic bacteria is about 100-1000 times less than that needed to kill bacteria in biofilms (reviewed by Edward & Kjellerup, 2013). The biofilm matrix presents enhanced resistance than the planktonic cells to microbes from different environmental stress and antimicrobial agents and this microbial population when found near the contaminated site can frequently degrade the pollutants formed in the contaminated sites with ease (Mitra & Mukhopadhyay, 2015).

The cell-cell communication using chemical signal molecules enables the bacteria in biofilms to coordinate gene expressions and behaviour of the entire community (Fuqua *et al.,* 2001; Solano *et al.,* 2014). These features have been reported to be associated with both Gram-negative and Gram-positive bacteria (Van Delden & Iglewski, 1998; Hentzer *et al.,* 2002).

Bacterial cultures growing in experimental microcosms (e.g. glass vials containing liquid growth medium) can form biofilms at the air-liquid (A-L) interface. These biofilms growing on the liquid surface or at the meniscus can be divided into different classes based on their physical strength, resilience and attachment. These includes the floccular mass (FM), physically cohesive (PC), viscous mass (VM) and waxy aggregate (WA) classes (Spiers *et al.,* 2006; Ude *et al.,* 2006). In addition to these four of A-L interface biofilms, visually obvious but limited growth in the meniscus region and attached to the vial walls has been referred to as meniscus biofilms (MB).

Table 6.1 Different classes of air-liquid (A-L) interface biofilms produced by environmental pseudomonads

Biofilm Attributes	Waxy aggregates (WA)	Floccular mass (FM)	Physically cohesive (PC)	Viscous mass (VM)
Occurrence	Rare	Common	Common	Common
Structure	Single-piece rigid and brittle structure	Multiple flocs	Single-piece flexible and elastic structure	Large viscous mass
Strength	Strong	Medium	Strong	Weak
Resilience	Good, disruption produces smaller fragments	Good, disruption produces flocs that are hard to destroy	Very good, hard to break into smaller fragments	Very poor, disruption solubilises the structure
Attachment	High	Medium	High	Poor
Matrix	No evidence for EPS, possible cell-to-cell interactions	Observed	Observed	Observed

Biofilm attributes includes; Strength, ability to withstand weight applied to the top of the biofilm; Resilience, response to applied physical disturbance such as gentle or vigorous mixing; Attachment, connection to the microcosm vial walls in the meniscus region; Matrix, evidence of EPS from behaviour of samples during microscopy (Source: Spiers *et al.,* 2006; Ude *et al.,* 2006). NB: The limited growth in the meniscus region and attached to the vial walls have been referred to as meniscus biofilms (MB).

The response of microorganisms towards chemical gradient through a process called chemotaxis has significantly contributed to bringing the cells in close contact with chemical pollutants (Pandey & Jain, 2002).

This process allows the cells to adsorb the chemicals to initiate growth through the formation of biofilms and surfactant production which leads to bioavailability of hydrophobic compounds and increased biodegradation rates (see reviews by Law & Aitken, 2003; Sampedro *et al.,* 2014). The robustness of biofilms linked with the diverse range of structural and metabolic characteristics make these communities beneficial in biofilm facilitated remediation of contaminated sites (reviewed by Edward & Kjellerup, 2013).

The genus *Pseudomonas* produces biofilms as a predominant form of growth on biotic and abiotic surfaces by synthesizing exopolysaccharide (EPS) composed of alginate that contribute to attachment and colonization of surfaces by *P*. *aeruginosa*, *P*. *fluorescens*, *P*. *putida* and *P*. *syringae* (Chang *et al.,* 2007; Orgad *et al.,* 2011). Though, in some instances alginate production may be affected by proteins, nucleic acids and high glucose content and other nutritional conditions which define its function as a constituent of the EPS (Czaczyk & Myszka, 2007; Parsek & Tolker-Nielsen, 2008).

Cellulose expression was also investigated for isolates used in this work, since it has long been recognised that some bacteria including *Pseudomonas* species produce cellulose which is a vital component of the extracellular matrix (Spiers *et al.,* 2006; Ude *et al.,* 2006). Celluloses are used in different biotechnology applications including bioremediation due to their wide structural diversity and

their physical, rheological and other inherent properties (reviewed by Edward & Kjellerup, 2013). Different components of the EPS were reported to facilitate irreversible attachment of microorganisms to hydrophobic and hydrophilic surfaces (reviewed by Vu *et al.,* 2009).

6.2 Research objectives

The aim of the research reported in this chapter was to investigate biofilms produced from pseudomonads (i.e. *Pseudomonas spp.*) isolated from activated sludge wastewater of a treatment plant and hydrocarbon contaminated soils samples recovered from a Sustainable Urban Drainage Systems (SUDS) site and to determine growth, strength and attachment levels, in relation to the biofilms formed.

The objectives of this research were:

1. To investigate and quantitatively characterise biofilm formation at the air-liquid interface of the 41 LSTRA isolates in static microcosms, including assaying for cellulose as the primary EPS;

2. To compare biofilm formation of the low-γ LSTRA isolates in static microcosms with growth in a column bead system;

3. To determine whether biofilm formation of the low-γ LSTRA isolates occurs at the oil-water interface.

6.3 Biofilms formation at the air-liquid interface

In order to investigate biofilm formation at the air-liquid interface, visual inspections were made at the meniscus region of the liquid column for turbidity and/or signs of growth and were classified by tipping the biofilm material onto Petri dishes (see Appendix A1. Table A1.4). (after Spiers *et al.* 2003).

6.3.1 Classification of biofilms

In order to classify biofilms formed at the air–liquid interface, *in situ* observation and inspection of material once tipped on Petri dishes was undertaken after Ude *et al*. (2006). The biofilms were subsequently classified as floccular mass (FM), physically-cohesive (PC), viscous mass (VM), waxy aggregate (WA) and meniscus biofilm (MB) based on a number of factors, such as strength (i.e. ability to resist load imposed on top of the biofilm), resilience (i.e. changes due to mixing), and attachment i.e. sticking to the microcosm vial walls at the meniscus region (see Table 6.1 for a description of these biofilm classes). Images of the two most common biofilm classes are shown in Figure 6.1. Biofiolm classifications for the 41 strains are given in Table 6.2. No waxy aggregate biofilms were observed.

6.4 Characterisation of biofilms using the combined biofilm assays

In order to quantitatively investigate growth, biofilm strength and attachment levels in static microcosms the combined biofilm assay were carried out (Robertson *et al*., 2013). Replicate KB microcosms (*n* = 8), was incubated

statically for three days before the combined assay. First, the strength of the biofilms formed was determined using the maximum deformation mass (grams) assay (Fig 6.4). Secondly, the mean growth of isolates in microcosms was determined by measuring the optical density (OD_{600}) of the cultures as shown in Fig 6.3. Finally, the determination of attachment levels of biofilms by measuring their absorbance (A) was undertaken as shown in Fig 6.5. In this assays, mean growth of between 0.833 – 2.058 OD_{600} was observed with mean strength ranging from 0 – 1.224 g and attachment levels of 0.220 – 2.830 A_{570}.

From the result of the combined assays, it was observed that all isolates grew well in the static microcosms but with weak biofilm strengths from the material seen in the growth media or from the result of the strength assay conducted similar to the work done by Robertson *et al.* (2013).

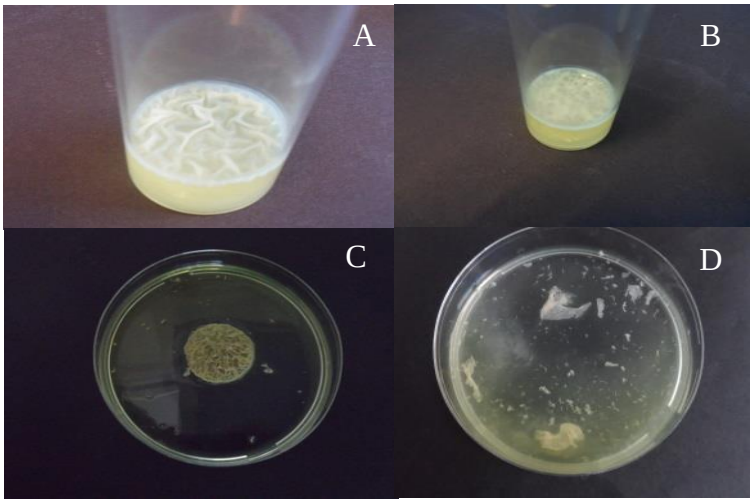


Figure 6.1 Biofilms formed at the A-L interface from pseudomonads are classified based on their full-bodied appearance and strength. Shown are the two most common biofilm classes observed, (A) the physically cohesive (PC) type biofilm, and (C) its material when tipped on Petri dish and (B) the floccular mass (FM) biofilm and (D) its material.

Table 6.2 Shown are the various classes of biofilms formed and cellulose expression amongst the isolates

Strains	Biofilm class	Cellulose expression
1	MB	–
2	PC	+
6	FM	–
8	PC	–
9	MB	–
63	MB	–
65	MB	–
69	MB	–
79	FM	–
83	MB	+
85	PC	–
86	MB	–
88	FM	+
94	FM	–
97	MB	–
101	PC	–
105	MB	–
106	PC	–
107	PC	–
111	PC	–
152	MB	–
166	MB	–
169	FM	–
175	PC	+
178	MB	–
179	MB	–
183	PC	+
190	MB	–
194	PC	–
195	MB	–
197	MB	+
211	MB	–
236	MB	–
239	MB	–
303	PC	+
327	FM	–
335	FM	–
336	MB	–
342	MB	–
354	MB	–
355	MB	–

MB, Meniscus biofilm, PC, Physical cohesive, FM, Flocullar Mass, (+), Positive, (-), Negative,

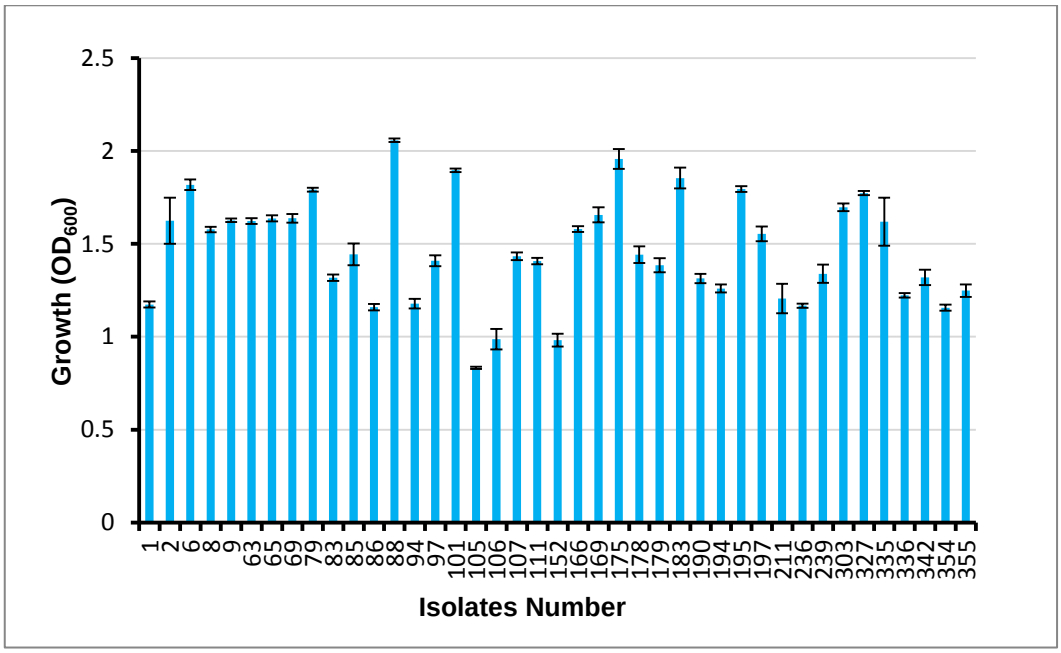


Figure 6.2 Pseudomonads showed significant differences in growth. Mean growth of each isolate from 3-day old static KB cultures was measured by their optical densities (OD_{600}) and many biofilms showed significant levels of growth. Mean ± SE of growth of each isolates were then plotted. The growth of between 0.832 – 2.058 (OD_{600}) were recorded. Isolates are indicted along the x-axis by their strain numbers, #1 - 355.

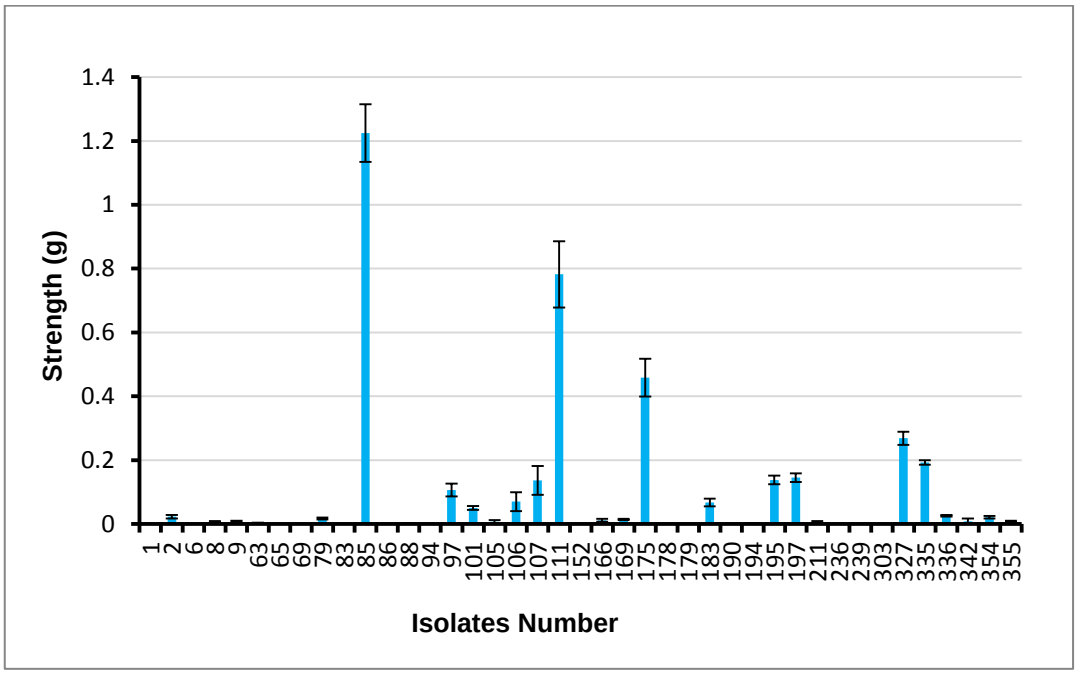


Figure 6.3 Pseudomonads produced biofilms with poor strengths. The mean strength of the biofilms was measured by maximum deformation mass (MDM) using three day old static KB microcosms. Mean ± SE of strength of each isolates were then plotted as shown. The strength of isolates ranges from 0 - 1.225 g. Many biofilms had no significant strength (i.e. did not retain a single glass ball of 0.0115 g). Isolates are indicted along the x-axis by their strain numbers, #1 - #355.

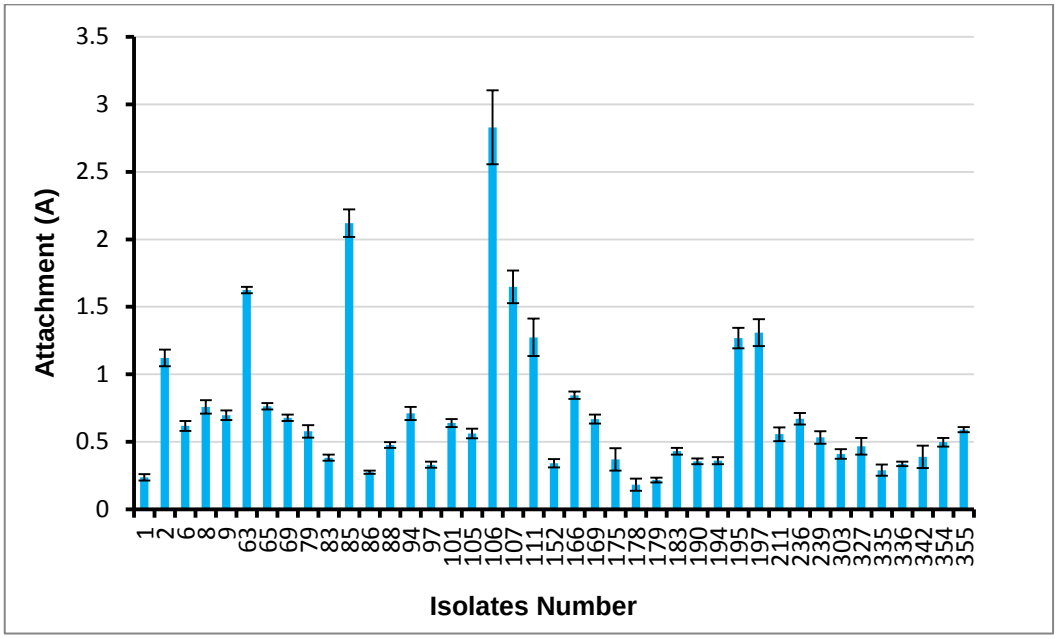


Figure 6.4 Pseudomonads showed significant differences in attachment levels. The mean attachment levels of each isolate from 3-day old static KB cultures were measured after staining with crystal violet. Mean ± SE of attachment of each isolates were then plotted as shown. The attachment levels of isolates ranges from 0.183 – 2.830 (A_{570}). Isolates are indicted along the x-axis by their strain numbers, #1 - #355.

6.5 Identification of cellulose in colonies

In order to investigate for cellulose expression amongst the *Pseudomonas* spp. bacteria, colony material was examined using calcofluor and flourescent microscopy (after Spiers *et al.,* 2003; Koza *et al.,* 2009). Out of the 41 isolates examined, 7 (17%) of the isolates were found to show significant amount of fibrous material viewed at 10x, 20x and 40x magnifications (after Spiers *et al.,* 2003; Ude *et al.,* 2006) as shown in Fig 6.5.

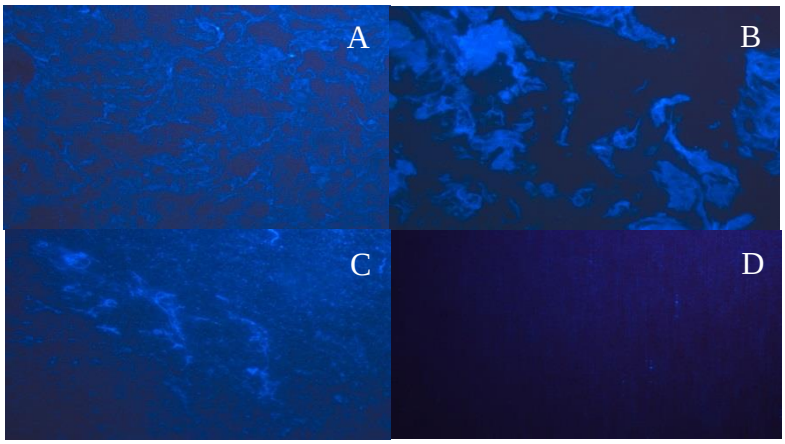


Figure 6.5 Cellulose expressions by pseudomonad isolates. Colonies were incubated on KB plates after which sample were stained with calcofluor for visualisation by fluorescent microscopy to confirm the presence of cellulose. Shown are images of epifluorescent microscopy taken at 10x, 20x and 40 x magnifications with cellulose positive strains (A, strain #2; B, strain #179; C, strain #88) and negative strain (D, strain #106).

6.6 Comparison of biofilm formation in static microcosms and glass bead columns

6.6.1 Growth in column bead system

In order to determine growth around the surfaces of stationary objects the glass bead columns was developed and this will also assess the environmental difference between the microcosm systems (i.e. planktonic, biofilm) and the glass bead systems (biofilm forming around the glass balls).

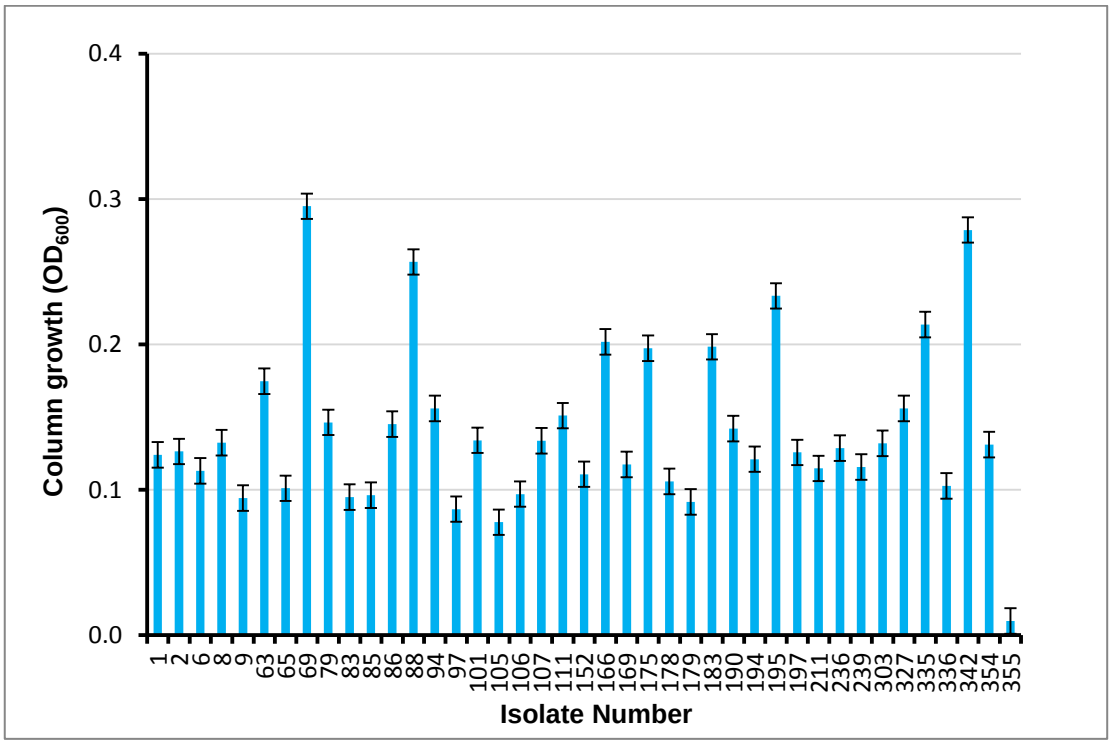


Figure 6.6 Pseudomonad Isolates in column system showed a significant level of growth. The mean growth (OD_{600}) of isolates from 3-day old static column system was measured. Mean ± SE of growth of each isolates were then plotted as shown. Mean growth of between 0.078 – 0.295 (OD_{600}) were recorded. Isolates are indicted along the x-axis by their strain numbers, #1 - #355.

6.7 Other correlations between growth, strength and attachment of biofilms formed from the combined assay

Biofilm development is dependent on growth, strength and attachment. Therefore, in order to determine the relationship between growth, strength and attachment levels of biofilms formed from the combined assay, pair wise correlation analysis was done. Significant correlation was observed between biofilm strength and attachment (r = 0.706; $P \leq 0.001$), and a weak correlation between growth and strength (r = 0.234; $P \leq 0.140$) and growth and attachment (r = 0.045; $P \leq 0.775$). Microcosm growth was significantly correlated with the column growth (r = 0.429; $P \leq 0.005$) and column growth was weakly correlated with biofilm attachment (r = 0.084; $P \leq 0.615$) and surface tension (r = 0.161; $P \leq 0.188$). Biofilm attachments was also found to be significantly correlated with surface tension (r = 0.475; $P \leq 0.002$) but weakly correlated with hydrophobicity (r = 0.126; $P \leq 0.429$), while the biofilm strength was moderately correlated with surface tension (r = 0.345; $P \leq 0.056$). Biofilm attachment was significantly correlated with surface tension (r = 0.226; $P \leq 0.002$) and the strength was moderately correlated with surface tension (r = 0.395; $P \leq 0.056$), as shown in Table 6.3. The pairwise correlation scatter plots of all biofilm parameters and other factors are presented in Appendix A4.

Table 6.3 Pairwise correlations of biofilm behaviour

Factor	Factor	r	*P*
Attachment	Surfacetension	0.475	0.002
Attachment	Hydrophobicity	0.126	0.429
Growth	Attachment	0.045	0.775
Column growth	Attachment	0.084	0.615
Growth	Strength	0.235	0.140
Column growth	Microcosms	0.429	0.005
Column growth	Strength	0.114	0.352
Growth	Surfacetension	0.063	0.712
Column growth	Surfacetension	0.161	0.188
Strength	Attachment	0.706	0.000
Strength	Surfacetension	0.395	0.056

P, significance value, r, correlation coefficient

6.8 Colonisation of oil surfaces by *Pseudomonas* spp. bacteria

In order to assess whether any of the 41 LSTRA isolates might be capable of colonizing used lubricating oil (ULO), a preliminary survey of biofilm formation was undertaken. This assay involves adding a drop of oil to water (or diluted growth medium) with a small culture medium and observing signs bacterial growth or potential biofilm growth for 3 – 5 days. The growth could be at the oil-liquid or oil-air or liquid-oil-air interface. Bacterial growth in the liquid was assessed by colour and opacity changes, whilst possible biofilm formation was identified at the oil-liquid interface by colour and biomass development.

All the 41 LSTRA isolates were assessed in this assay. For most of the isolates tested, no signs of biofilm development were seen (e.g. Strain #354, Fig. 6.7(D), though some growth in the liquid was observed. However for several other isolates examined, clear changes of colour at the air-oil-liquid were observed as a white line (See Fig. 6.7 A-C).

In order to determine whether the white line resulted from bacterial growth and biofilm development (i.e. biomass) or enzymatic or chemical changes to the oil surface, the same experiment were performed using cell-free culture supernatants and a summary of the results are shown in Table 6.4. This suggests that the colouration of the oil was probably due to bacterial metabolism rather than development of biofilms.

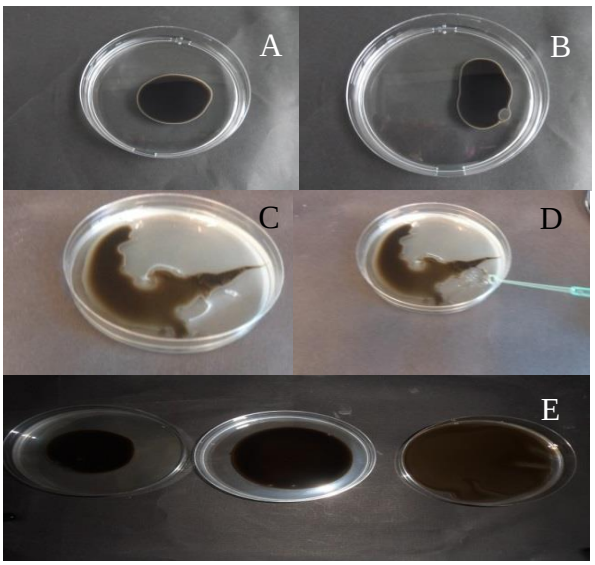


Figure 6.7 Pseudomonads producing a white line around oil droplets. Shown are the developments of white line around oil drops of used lubricating oils, A-B. The white line was gently separated from the oil, C-D. Growth was observed but no white line around the oil droplet 'E'.

Table 6.4 A Summary of treatments to test for growth and biofilm development or chemical changes of bacterial (pseudomonads) cultures in oils

Treatment	Growth	Result
Washed cells	No	No white line
Washed cells + media	Yes	White line
Cell-free supernatant	No	White line
Media	No	No white line

6.9 Discussion

The overall aim of the work presented in this chapter was to determine whether the pseudomonad isolates produced biofilms as an indication of whether they might be used in bioremediation and other biotechnology applications in the future.

The 41 LSTRA pseudomonad isolates were investigated for biofilm formation in static microcosms for 3-days after selection on plates and results showed A–L biofilms of considerable phenotypic variation, which is predominantly found among environmental *Pseudomonas* spp. (Ude *et al.* 2006), including 7 (17%) of 41formed floccular mass biofilms and 23 (56%) of 41 meniscus biofilms, while 11 (27%) of 41 are physically cohesive biofilms. Biofilms have been reported to play a key role in supporting bioremediation of hydrocarbon contaminated soils (Singh & Cameotra, 2004; Singh *et al.,* 2006). It has also been reported in many studies that bacteria move (swimming) towards chemical pollutants within sediments by a process known as chemotaxis and attaches to the surfaces of the substrate possibly through the formation of biofilms (O'Toole & Kolter, 1998; Watnick & Kolter, 1999).

Significant growth was observed in the biofilms produced ranging from 0.833–2.058 (OD_{600}) with weak strength ranging from 0–1.224 g and attachment levels of 0.237–2.830 A_{570}, were observed, similar to what was obtained in Robertson *et al.* (2013), who investigated a set of sixty randomly picked psychrotrophic pseudomonads from spoilt vacuum packed meat.

The matrix components of the LSTRA pseudomonad isolates was sought through identification by microscopy and a total of 17 % (7/41) isolates were identified as expressing cellulose. Peudomonads are known to express a range of different EPS involved in biofilm formation including alginate PEL (a glucose endowed polymer) and PSL (pentasaccharides containing d-mannose, d-glucose and rhamnose), these polysaccharide are usually regarded as a shared resource that provide benefit to the biofilm community by preserving structure, supporting signalling, and protecting the organisms involved from predation, competition, and environmental stress which might be utilized by the non-cellulous expressing isolates tested here (Davey & O'Toole, 2000; Watnick & Kolter, 2000). This proportion was similar to what was reported in Ude *et al.* (2006), who investigated 140 biofilm-forming isolates and found 28 to express detectable levels of cellulose. The comparative study of biofilm formation in static microcosms and in column bead systems showed a significant growth relationship in the two environments ($r = 0.429$; $P \leq 0.005$). This suggests that these strains are likely to be able to form biofilms in natural environments as well. Although biofilm formation was tested using oil droplets, no evidence was found to support this for any strain tested. This might be due to the short duration in testing the isolates for biofilm formation at the oil interface. Longer incubation period of observation might result in the production of biomass or the bacteria do not form biofilms (i.e growing somewhere in the medium) (Crouzet *et al.,* 2014).

6.10 Conclusion

The research work presented in this chapter showed that a substantial number of the pseudomonad isolates formed biofilms at the air-liquid interface in static microcosms. Different classes of biofilms were observed, including floccular mass, physically-cohesive and viscous mass with an additional class of biofilm, the meniscus biofilm also observed. The strains showed significant different levels of growth in the static microcosms and column microcosm systems, with most static microcosm biofilms having little significant strength or attachment.

Few of the isolates expressed cellulose as the primary EPS involved in biofilm development in pseudomonads. The growth in static microcosms and the column bead systems showed a significant relationship in the two environments i.e. growth occurs as free moving, biofilms as well as on stationary objects.

Finally, it was observed that most of the isolates discoloured oil droplets, though no evidence of biofilm formation at the oil-liquid-air interface was observed. The discolouration might also be due to dissolution and assimilation of carbon from the ULO by bacterial cells. Further studies need to be carried out to unravel the reactions between the media and the used lubricating oil.

Chapter 7

Discussion, conclusions and suggestions for further work

7.1 Aims and objectives of this research

The overall aims of the research work presented in this thesis were to isolate and phenotypically characterise pseudomonads and *Pseudomonas*-like isolates from activated sludge wastewater and soil samples from road side drainage, including assessing strain diversity and surfactant production. Thereafter to identify pseudomonads that significantly lower the surface tension of cultures by statistical means and to predict the minimum surface tension γ_{Min} reducing activities of pseudomonad isolates by quantitative tensiometry. Then to determine whether the set of 41 low-γ liquid surface tension reducing activity (LSTRA) isolates represents a diverse collection of strains using the phenotype data, and to assess their behaviour using foam stability, emulsion and oil-displacement assays.

Evidence to support chemical variation within the types of surfactants expressed by isolates producing very similar surface tensions was pursued as this could be used to select isolates for further analysis and testing of novel biosurfactants. Finally to characterize biofilms produced by *Pseudomonas* spp. in experimental microcosms, examine cellulose expression as the primary

extracellular polymeric substances, (EPS) and to determine whether biofilm formation occurs at the oil-water interface.

7.2 Isolation and characterization of pseudomonad bacteria expressing biosurfactants

Isolation and phenotypic characterization of bacteria is a key step to identification of strains for further analysis. *Pseudomonas* species are found in almost all environments, i.e. air, water and soil (Romanenko *et al*., 2015; Taguett *et al*., 2015). In this work, 355 pseudomonads bacteria from activated sludge wastewater and soils from road side drainage (SUDS sites) were isolated. Although isolates were selected using PSA+CFC agar, they were not identified to species level using either metabolic profiling or 16S rDNA sequence analysis. Though, the confirmation of these isolates to species level is very necessary in order to identify or find closely associated isolates to a particular species to which it belong for future study where a particular isolates or species might be suitable for a particular biotechnological applications and that will also form basis for which the genes could be manipulated to improve it's properties for specific function or use.However phenotypic characteristics of the isolates have shown that they possess characteristics similar to pseudomonads (Prakash & Irfan, 2012).

Moreover, study by Yamamoto *et al.* (2000) has shown that, genus pseudomonads identification to species level cannot be resolved completely using 16S rDNA sequencing and the DNA-DNA hybridization methods and this

not unconnected with the slow rates of evolution of 16S rDNA and the ineffectiveness of DNA-DNA hybridization method to also estimate the exact distance between the distantly related species.

A promising method was proven using the phylogenetic clustering of *Acinetobacter* strains based on *gyr*B sequence analysis which is similar to the genomic species delineated by DNA-DNA hybridization and the results obtained from this analysis using the *gyr*B and *rpo*D sequence may close this resolution gap between 16S rRNA sequence analysis and the DNA-DNA hybridization earlier technique used for delineation of bacterial species (Yamamoto *et al.,* 2000).

In this research we are interested in surfactant producing *Pseudomonas* that may have potential use in biotechnological applications with less biological replication without confirming the species to which the isolates belongs.

Hierarchical cluster analysis of the results of the phenotypic assays indicates high variation or diversity amongst the isolates as generated by the dendogram. This analysis was further clustered into 54 groups based on similarity. This type of analysis follows that used previously in a study conducted on *Pseudomonas* isolates recovered from vacuum-packed venison which divided isolates into groups based on a similar set of phenotypes (Robertson *et al*., 2013).

Catalase and oxidase production from *Pseudomonas* spp. is one of the most important tests that differentiate microorganisms by way of their enzymatic activity (Ugur *et al.,* 2012). The phenotypic characterization of the collection of pseudomonads detected 243 (68%) of the 355 isolates to be oxidase positive

and 109 (31%) catalase positive. This result obtained is in agreement with the investigations carried out based on different morphology and reactions on KB plates detected the organism to be Gram negative, rod-shaped, oxidase and catalase positive confirming the organism to be *Pseudomonas* spp. (Meliani & Bensoltane, 2014).

Catalase enzymes produced by microorganisms helps in breaking down hydrogen peroxide and convert it into water and oxygen. This enzymes is increasingly been used in many industrial processes such as in bleachng processes (i.e textile and pulp) to convert toxic and carcinogenic waste products into a less harful substances before being dischaged into the environment (Jegannathan & Nielsen, 2013). For example *T. brockianus* catalase enzymes produced by cellular activity helps in protecting the organisms from oxidative stress by interfering in the subsequent dying steps in textile industry (Thompson *et al.,* 2003).

Therefore identifying the genes amongst the isolates in this work producing enzymes catalase would have potential applications especially when industrially relevant volume is required, so that the genes that encode the catalase production can be inserted into a microbe that is easily grown in large quantities.

While oxidase tests are also used to confirm bacteria (i.e. pseudomonads) that produce cytochrome c oxidase, an enzyme that is involved in electron transfer in bacteria and It is also used in characterization of bacteria into oxidase

positive or negative, as evidenced in this work. Catalase enzymes are now gaining attention due to their antioxidative functions and could be of used in a number of biotechnological applications, such as in food and medicinal industries where antioxidative activity is required and also for bleaching in textile industry (Pote *et al.,* 2014).

Microbial lipases are increasingly been used as bio-catalysts in different industrial processes due to their diversity and versatility and are preferred over that secreted by plants and animals owing to their broad substrate specificity, higher stability and low production costs (Pote *et al.,* 2014). Here, in this work 126 (35%) of the 355 isolates from wastewater and soil environment produced the enzyme lipase. These lipase-producing pseudomonads could be used in the hydrolysis of organic pollutants as the lipase aid the degradation and utilization of the organic polymers (Karigar & Rao, 2011). They find useful applications in the bioremediation of oil spills. An example is their use in evaluating degradation of freshly oil-polluted soils by monitoring the decontamination in the laboratory in which the lipase activity and counting of oil-degrading microorganism at time intervals were correlated with the levels of hydrocarbon concentrations in the soil (Margesin *et al.,* 1999). They also found useful applications in biotechnology because of their diverse nature, such as in food industry, feed supplements, biomedical science and chemical industry (Sánchez-Porro *et al.,* 2003; Karigar & Rao, 2011).

In this work, the phenotypic characterization of the 355 strains showed that 251 (78%) of the 355 isolates secrete enzyme protease. This showed that substantial numbers of pseudomonad isolates secrete the enzyme protease.

Proteases are microbial enzymes known to hydrolyze peptide bonds (proteinaceous substances) in aqueous environment and synthesize them in non-aqueous environment (Karigar & Rao, 2011). These enzymes could be used in different biotechnology applications.

It has also been reported that microbial extracellularly produced enzymes has gained acceptance in the enzyme market globally, representing about 60% sold worldwide and is now increasingly been used as biocatalysts in many industrially relevant biotechnological applications, involving different industrial processes of great benefits to mankind, including baking, brewery, dairy, detergents, pharmaceuticals, leather tanning, and in cooking meat (i.e. tenderization) to make it palatable (Najafi, 2005; Jyothi & Rao, 2009). The substantial number of protease enzyme secreting isolates obtained in this work could provide potential candidate proteases for biotechnology applications. The activity of *Pseudomonas fluorescent* isolated from a local red soil was monitored for its ability to utilize diesel oil and glycerol as the sole carbon source and the extracellular enzymes that accompanied it during the utilization process and was found to be protease and urease (Jyothi & Rao, 2009). It is likely that protease for example, is used to digest larger moleculer weight proteins, perharps from decaying plants, fungal and animal material, to provide nutrients for bacterial growth. This suggests that the growth and activity of bioremediation of agents (such as the protease-positive isolates found here) could be improved by the addition of protease-based nutients to the contaminated soil during bioremediation.

For motility tests, out of the 355 isolates it was confirmed that 184 (52%) to be swimming, 117 (33%) swarming and 60 (17%) twitching motility positive. This result consolidated our search for pseudomonads and *Pseudomonas*-like bacteria as this is consistent with the work done by Kohler *et al*. (2000); Deziel *et al*. (2003) in which the swarming motility displayed by *P. aeruginosa* was connected to the presence of rhamnolipid surfactant in addition to swimming and twitching motilities demonstrated. Pseudomonads are known to exhibit different types of motility and this is one of the main features of identifying *Pseudomonas* spp. Swimming and swarming motilities are forms of movements driven by the use of flagella either as individual cells moving in liquid environment or as multicellular bacterial movements on surfaces, while twitching motility is a surface movement that occurs as a result of extension and withdrawal of type IV pili (Harshey, 2003; Kearns, 2010).

This showed that substantial number of the pseudomonads isolated for this work have the potentials of moving towards chemical contaminants or towards a surface to initiate biofilms. It has been reported by many researchers that bacteria responds to chemical nutrient by movements (i.e. swimming, swarming and twitching) for growth and survival, which is an important feature to initiate or effect bioremediation of contaminants in a process known as chemotaxis (Kaiser, 2007; Kearns, 2010).

Heavy metals are found naturally in the environment or through man's activities (anthropogenic) and different organisms show different resistance towards antibiotics. These pose a serious problem to the environment and to public health due to their persistence and build-up (see reviews by Bruins *et al.,* 2000;

Dixit *et al.,* 2015). Microorganisms inhabiting soil, water and sediments normally live in close association with these metal either as a basis of their biological activity in utilizing these metals (i.e. Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, V, Zn). They could also be in the form of nonessential, non-toxic metals e.g. Cs, Rb, Sr and T or toxic such as Ag, Al, Au, Cd, Hg, Pb, Sn and Ti, present in the environment and as a result of this, different microbes have developed survival mechanisms to adapt to these metals by the use of chromosome, plasmid, or transposome encoded methods of resistances (see reviews by Bruins *et al.,* 2000).

Although most of these resistances are associated with plasmid and are highly specific to the functions of the cations and anions present. Therefore metals play a vital role in the life of microorganisms with respect to the environment where they live and the understanding of how microorganisms resist metals can be used to develop strategies on how their detoxification and removal from the environment be successfully achieved (see reviews Bruins *et al.,* 2000).

In this work, resistances of isolates towards heavy metal mercury was assessed as an aspect of phenotypic characterization of the isolates and found 306 (86%) of the 355 to harboured resistance to mercury. Bacteria develop resistance to heavy metals in order to survive the environment exposed to heavy metal contamination and the resistance differs from one organism to the other due to the composition of their plasmids (Bruins *et al.,* 2000; Malik & Jaiswal, 2000). By testing isolates for the appropriate heavy metal resistances, it should be possible to choose more appropriate isolates as bioremediation agents to fit

specific environmental conditions where multiple toxins might be present during which the priority pollutant targeted for bioremediation.

Resistance to antibiotics, tetracycline and kanamycin of the 355 isolates were tested and found 307 (86%) tetracycline and 276 (78%) kanamycin positive. This showed that a large proportion of the isolates were resistant to levels of kanamycin and tetracycline used. This confirmation is in agreement with the study reported by Azimi & Cephane, (2015) who found out that all the 61 *Pseudomonas* spp. isolated from hospital ward surfaces were resistant to penicillin, cephalexin and vancomycin but responded differently to other antibiotics. Interestingly, this study is also in harmony with the work by Nikolouli & Mossialos, (2015) which showed that *Pseudomonas entomophila* exhibits antibiotic resistance toward several antibiotics including ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline. Although the antibiotic resistances were checked for the isolates examined here, the primary reason for doing so were to investigate further diversity between isolates, and secondly to see whether some were sensitive to kanamycin and tetracycline. These two antibiotic resistances are commonly used in the genetic modification of pseudomonads. The identification of potential bioremediation strains with kanamycin and tetracycline sensitivity would allow them to be further manipulated to make them better bioremediation agents (e.g by inserting genes to make use of exogenously added nutrients, i.e. a secreted protease, etc.)

The biodiversity among the 355 isolates utilizing sugar and producing organic acids was assessed because *Pseudomonas* spp. are known to utilize carbohydrates (sugar), or a number of carbon compounds (substrate) as the

sole carbon source for growth and subsequently produce acids as end products (Agrawal *et al.,* 2015). This is one of the basis for which pseudomonads could be identified. A high proportion of the isolates, 297 (84%) of the 355 isolates utilized sugar and 292 (82%) produced organic acids as end products. Strains of *Pseudomonas* were able to grow and be detected using several carbohydrates and produce organic acids like glucose, mannose, mannitol, gluconate, capric acid, malic acid, citrate, phenylacetic acid, octanoate and citrate as sole carbon source (Meliani & Bensoltane, 2014). This organic acids produced are commonly refined and have useful biotechnology applications in the production of bio-fuels as well as building-block chemicals to shift from reliance on petroleum to the use of renewable resources (Sauer *et al.,* 2008).

Although the substrate utilization of the isolates examined here has not been further developed, it is possible that the specific isolates may be able to grow and show higher bioremediation activity in certain environments where key nutrients are available than other isolates which might require a different set of nutrients. These characteristics could be used by supplying specific nutrients during the bioremediation process (e.g. a specific sugar or acid) to improve the growthof the bioremediation agent, or by genetically modifying the isolate to be able to utilize a substrate it could not use before.

More so, the surfactant producing isolates where subjected to biofilm formation at the air-liquid interface, including identification of cellulose and colonization of oil surfaces Different classes of biofilms were observed, including floccular mass, physically-cohesive and viscous mass with an additional class of biofilm, the meniscus biofilm also observed. The biofilm forming ability of *Pseudomonas*

species observed in this study is in conformity with findings by Ude *et al*. (2006) and Koza *et al.* (2009) who found various classes of biofilms formed at the A-L surfaces of static microcosms. Few of the isolates expressed cellulose when viewed under microscopy. This study is in conformity with that of Ude *et al*. (2006) who reported that cellulose expression and biofilm formation are common amongst diverse environmental pseudomonads. Microbes have been reported to produce biofilm matrix that contains polysaccharides as an integral part of the formation of bacterial communities (Limoli *et al*., 2015). The comparative study of biofilm development in static microcosms and the column bead systems showed a significant growth relationship in the two environments i.e. growth occurs as free moving biofilms as well as on stationary objects. This is in line with the work reported by Udall *et al*. (2015) who compared the different aspects of wrinkleality of a Wrinkly Spreader in both drip-fed columns and static microcosm that evaluated microcosm growth, biofilm strength and attachment, as well as fitness in static microcosms.

Polysaccharide production in environmental *Pseudomonas* isolates has also been shown to be important for colonization of plant tissues, soil and fresh water streams. The role of these polysaccharides includes basic functions such as maintaining the structural integrity of the cell envelope, protection against adverse conditions or host defence mechanisms and/or storage of nutrients, as well as more complex functions such as facilitating aggregation, adherence and interactions within bacterial communities.

The abilty to form biofilms on surfaces such as soil particles near to aggregation of hydrophobic organic contaminants (eg. oil or residues in the pore network of

soil sediments) might improve the activity of a bioremediation agent. Biofilm formation might prevent the bacteria from competing with local bacteria, protection from some toxic components present in the contaminants, and allowing the expression of surfactants, lipase etc. to be more effective than if they were produced by single cell.

Surfactants are also known to play a major role in maintaining biofilms (Pamp & Tolker-Nelson, 2007). However, it was also reported to hinder and breakdown of biofilms with two *Pseudomonas putida* lipopeptides surfactants (Kuiper *et al*., 2003). It was also observed that most of the isolates discoloured oil droplets, though no evidence of biofilm formation at the oil-liquid-air interface was observed. The change in the colour of the used lubricating oil observed might due to the bacteria using the carbons present in the oil for growth (Sawadogo *et al.,* 2014).

It was also found out from the test that 71 (20%) of the 355 isolates expressed surfactants by the drop collapse method (Chen *et al*., 2007). This is one of the reliable and simple preliminary tests conducted to assess microorganisms for the production of biosurfactants before further confirmation by quantitative tensiometry. Biosurfactants are surface active compound produced by microorganism that lowers the interfacial tensions between air-liquid, liquid-liquids and liquid-solid.

7.3 Predicting the minimum surface tensions (γ_{Min}) of bacteria expressing biosurfactants

The ability of microorganisms to reduce surface tension of liquids has been reported by many researchers and most prominent amongst them are the ones reported by Morikawa *et al.* (1993); Nielsen *et al*. (2002); Fechtner *et al*. (2011); Xia *et al*. (2011); Saimmai *et al*. (2012) who found that the surface tension cannot be reduced to below 22 – 25 $mN.m^{-1}$. However, these studies illustrate a fundamental problem with all such surveys that are focused on identifying better isolates. In each case a selection of surfactant expressing isolateswas made from o larger collection of bacteria, but no effort was made to determine whether these were the best that could be isolated from the environments tested (i.e if 100 more strains were tested, would a better one be found?). This type of problem can be addressed by the appropriate statistical-based prediction of surfactant activity (i.e Minimum surface tension, γ_{Min}) (Fechtner *et al.,* 2011). Furthermore, some studies use single measurements of surface tension with no indication of reproducibility (Xia *et al.,* 2011).

In an analysis of 72 surfactant-producing pseudomonads by Fletchner *et al*. (2011) predicted the minimum liquid surface tension (γ_{Min} of 24.16 $mN.m^{-1}$). This work presented here confirm minimum using a collected of different pseudomonads isolated from activated sludge wastewater and SUDS soil.

Although my findings confirmed the limit suggested by Fechtner *et al*. (2011) the theoretical limit determined in this work solely applied to KB culture supernatants, there might be possibility of increasing the limit because bacterial

surfactant expression may be opposed by stronger agents such as pH, salts and other metabolites (Ayed *et al.,* 2015; Lan *et al*., 2015). It is suggested that further study of the ability of bacteria to reduce surface tension should be focused on purifying the surfactants produced and at the same time determining the corresponding critical micelle concentration (CMC) point.

7.4 Demonstration of biosurfactant behaviour of a homogenous class

Several studies confirmed that different species of *Pseudomonas* produced different types of surfactants (Kügler *et al*., 2015; Liu *et al*., 2015) and this might either be cationic, anionic, amphoteric and nonionic depending on the charge carried by their polar groups (Abdel-Mawgoud *et al*., 2010). Surfactants can also exhibits both cationic and anionic midpoints committed to the same molecule. These charges might also influence the chemical behaviour of the surfactants produced (Abdel-Mawgoud *et al*., 2010). Here in this work, it was observed that substantial number of the isolates significantly reduced the surface tension of sterile King's B (KB) medium from 52.8 ± 0.5 $mN.m^{-1}$ to between 24.5 ± 0.1 $mN.m^{-1}$ and 49.1 ± 1.4 $mN.m^{-1}$.

This is consistent with the work reported by Meliani & Bensoltane (2014), in which *P. aeruginosa, P. fluorescens,* and *P. putida* reduced the surface tension of liquid cultures ostensibly by biosurfactant production from 75 $mN.m^{-1}$ to 55 $mN.m^{-1}$, 45 $mN.m^{-1}$ and 36 $mN.m^{-1}$.

The behaviour of surfactants expressed by the isolates examined here was studied using foam stability, emulsion and oil displacement assays and significant differences between the isolates were observed in all the tests

conducted (ANOVA, $P < 0.001$), this showed that the 41 low-γ (LSTRA) pseudomonads isolates might be exhibiting different chemical behaviour. This is in conformity with findings of the study on the chemical behaviour of surfactants produced by microorganisms and results showed that a particular type of surfactant might be important for specific use or application (Sanchez *et al.,* 2007; Zhong *et al*., 2008; Banat *et al.,* 2010). The pairwise correlations of the surfactant behaviour assays investigated in this work showed that 5 of the 15 correlations are found to be significant ($P < 0.001$). The low number of statistically significant positive correlation of surfactant behaviour might be attributed to the release of different surfactant concentrations in the media (Meliani & Bensoltane, 2014).

7.5 Suggestions for further work

There is the need to identify the isolates to species level by conventional and molecular techniques. Further work could be undertaken to identify the surfactant expressing isolates of interest to species level using conventional biochemical sequencing techniques. For example, substrate utilization kits (eg. EPI tests) could be used to determine methabolic profiles and this is used to identify isolates with the *Pseudomonas* genus and 16S rRNA sequences obtained to allow a phylogenetic identification to be undertaken. The value of this would be to allow the identification of isolates having strong similsrity to strains already characterized in terms of metabolic requirements, biochemical potential, and risks to human health and to the environment.

Further work can also be undertaken to modify select isolates to improve their ability to survive in contaminated soils and sediments by inserting exogenous genes (e.g secreted proteases) or improving the expression of fuctions already present (e.g lipase).

In order to investigate the nature of the limit to surface tension, it will be important to purify key surfactant for test in different pH and salt buffers.

Finally, future research could aim to test selected isolates or genetically modified version isolates in real environments in which bioremediation is required. This would be potentially interesting as it could allow an evaluation of the phenotypic and surfactant characteristics established with the very real constraints the isolates would have in contaminated environments.

7.6 Final concluding statement

Environmental pseudomonads have been isolated and characterized following standard microbiological techniques and shown to be of diverse collection by Hierarchical cluster analysis. The biosurfactants producing ability of environmental pseudomonads have been surveyed and determined using the drop collapsed assay and tensiometry. The minimum liquid surface tension reducing activity (LSTRA) was subsequently determined using the Individual Distribution Identification (IDI) analysis, which signified a fundamental limit to the reduction of liquid surface tension of bacterial cultures. The behaviour of biosurfactants in different media was assessed by foaming, emulsion and oil displacement assays and significant differences amongst the isolates were also

observed. A subset of isolates exhibiting similar surface tension but different chemical structures was identified and evidence to prove these was also established and finally, the biofilm forming ability of the LSTRA isolates in an experimental microcosms, cellulose expression and colonization of oil surfaces was also assessed.

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Appendices

Appendix A1

Additional information for chapter 3

Table A1.1 Previous chemical analysis of soil samples collected from Swale 9 (2008-2012)

Sample points	Period	As	Cr	Cu	Pb	Ni	Zn	Cl	Ph	Oil
		mg/kg								
Sw9 inlet	2008	7.8	15.6	12.6	31.8	13.9	47.0	-		184.0
Sw9 inlet	2009	6.7	28.0	38.0	47.0	21.0	170.0	-	19.0	720.0
Sw9 inlet	2011	8.1	29.0	35.1	32.4	19.5	73.5	-	-	-
Sw9 inlet	2012	7.2	46.5	139.0	76.7	25.6	877.3	3570.0	132.6	1780.0
Sw9 outlet	2012	6.4	29.0	54.8	33.7	18.0	293.0	201.0	10.4	1770.0

Note: As, Arsenic, Cr, Chromium, Cu, Copper, Pb, Lead, Ni, Nikel, Zn, Zinc. In 2012, soil samples collected and analysed at the inlet of swale 9, showed Cu, Zn were above the threshold limits. The TPH (Oil) i.e. Total Petroleum Hydrocarbon at the inlet and outlet of swale 9 were high above the threshold limits.

Table A1.2 Summary of chemical analysis of soil sample collected for this work from Swale 9 outlet (13-09-2012)

Outlet Samples	Period	As	Cr	Cu	Pb	Ni	Zn	Oil
		mg/kg						
Swale 9.1	2012	7.2	53.3	148.5	86.2	28.4	747.3	2680.0
Swale 9.2	2012	5.2	24.7	30.4	22.0	15.1	158.4	1310.0
Swale 9.3	2012	6.4	31.6	31.6	35.9	25.4	117.0	638.0

Note: As, Arsenic, Cr, Chromium, Cu, Copper, Pb, Lead, Ni, Nikel, Zn, Zinc. The amount of chemicals found at three different locations of the outlet are shown.

Table A1.3 Sediment thresholds values for special wastes

Parameter (mg/kg)	CLEA	OPSQL	UK ICRCL
Arsenic (As)		10	33
Cadmium (Cd)	-	15	10
Chromium (Cr)	130	1000	110
Copper (Cu)	-	130	110
Lead (Pb)	450	2000	250
Nickel (Ni)	50	70	75
Zinc (Zn)	-	300	820
pH (pH unit)	-	>5	-
Oil (TPH)	-	1000	1500
Orthophosphate (P)	-	-	2000
Ammoniacal Nitrogen	-	-	4800

CLEA, Contaminated land exposure assessment, OPSQL, Ontario provincial sediment limit, UK ICRCL, United Kingdom interdepartmental committee on the redevelopment of contaminated land.

Table A1.4 A summery of different types of biofilm formed at the air-liquid interface

Biofilm types	N	(%)
Floccular mass	79	22
Physical cohesive	52	15
Meniscus biofilm	147	41
Viscous mass	13	4
Waxy aggregates	3	1
No biofilm	61	17
Total	355	100

N, number of visible type of biofilm formed from the total collection of 355 strains, %, percentage of the of each type biofilm formed

Table A1.4 Summary of the phenotypic characterisation of isolates from activate sludge wastewater and SUDS soil

Origin	Phenotype																											
	Oxidase			Catalase			Surfactant			Siderospore			Swimming			KB Culture Acidity			Sodium chloride Tolerance			Swarming Motility			Twitching Motility		
	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%
Wastewater	252	162	*64*	252	100	*40*	252	63	*25*	252	116	46	252	155	62	252	0	0	252	0	0	252	96	*38*	252	51	*20*
Soil	103	81	*97*	103	9	*9*	103	8	*8*	103	18	17	103	29	28	103	0	0	103	0	0	103	21	*20*	103	9	*9*
Total	355	243	68	355	109	31	355	71	20	355	134	64	355	184	52	355	0	0	355	0	0	355	117	33	355	60	17

N, number, P, positive, %, percentage positive

Table A1.5 Summary of the phenotypic characterisation of isolates from activated sludge wastewater and SUDS soil

Origin	Phenotype																							
	Lipase Secretion			Gelatinase Activity			Sugar Utilization			Protease Secretion			Acid Prd in Maltose			Mercury Resistance			Tetracycline Resistance			Kanamycine Resistance		
	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%	N	P	%
Wastewater	252	75	*30*	252	217	*86*	252	207	*82*	252	197	*78*	252	206	*82*	252	218	*87*	252	220	*87*	252	193	*77*
Soil	103	51	*50*	103	86	*83*	103	90	*87*	103	54	*52*	103	86	*83*	103	88	*85*	103	87	*84*	103	83	*81*
Total	355	126	35	355	303	85	355	297	84	355	251	71	355	207	58	355	306	86	355	307	86	355	276	78

N, number, P, positive, %, percentage positive

Table A1.6 Summary of the phenotypic characterisation and clustering of pseudomonad isolates recovered from activate sludge wastewater and SUDS soil

This is presented in the subsequent pages;

Strain	Site	Sider	Bc	Oxid	Cat	Surf	Swim	Mucd	Cuac	Nacl	Swar	Twit	Lsec	Gel	Sug	Prot	Acpr	Hg	Tet	Kan	Clst-54
#1	ww1	p	mb	p	P	p	p	p	n	n	p	n	p	p	p	p	p	p	p	p	1
#2	ww1	p	pc	n	n	p	p	p	n	n	p	p	p	p	p	p	p	p	p	p	6
#3	ww1	p	mb	p	n	n	n	p	n	n	p	n	p	p	p	p	n	n	p	p	11
#4	ww1	p	pc	n	n	n	p	p	n	n	n	n	n	p	p	p	p	n	n	p	2
#5	ww1	p	mb	p	p	p	p	p	n	n	p	n	p	p	p	p	p	p	p	p	1
#6	ww1	p	fm	p	n	p	p	p	n	n	p	p	p	p	p	p	n	n	p	n	31
#7	ww1	p	pc	p	n	n	n	p	n	n	p	n	p	n	p	p	n	n	p	p	11
#8	ww1	p	pc	p	p	p	p	p	n	n	p	n	p	p	p	p	n	p	p	p	1
#9	ww1	p	mb	p	p	p	p	p	n	n	p	n	p	p	p	p	n	p	p	p	1
#10	ww1	p	mb	n	n	n	n	p	n	n	p	n	p	n	p	p	n	n	n	p	11
#11	ww1	p	mb	p	p	n	p	p	n	n	n	n	n	p	p	p	p	n	n	n	2
#12	ww1	p	pc	p	n	n	p	p	n	n	p	n	p	n	p	p	p	n	n	n	35
#13	ww1	p	fm	p	n	n	p	p	n	n	p	p	p	p	p	p	p	p	p	p	50
#14	ww1	p	pc	n	n	p	p	p	n	n	p	n	n	n	n	n	p	p	p	n	7
#15	ww1	p	fm	n	n	n	n	p	n	n	n	n	n	n	p	n	n	p	n	p	3
#16	ww1	p	fm	p	n	n	n	p	n	n	n	n	p	p	p	p	n	p	n	p	4
#17	ww1	n	fm	p	n	p	n	n	n	n	n	n	p	n	n	p	n	p	n	p	4
#18	ww1	n	fm	p	p	n	n	p	n	n	n	n	p	p	p	p	n	p	p	p	4
#19	ww1	n	fm	p	n	n	n	p	n	n	n	n	n	p	p	n	p	p	n	n	20
#20	ww1	n	fm	p	p	n	n	n	n	n	n	n	n	p	p	n	n	n	n	n	15
#21	ww1	n	fm	p	p	n	p	p	n	n	n	n	n	p	p	n	p	p	p	n	8
#22	ww1	n	fm	p	n	n	p	n	n	n	n	n	n	p	p	p	p	p	n	p	5
#23	ww1	n	fm	n	p	n	n	n	n	n	n	n	p	n	p	p	p	p	p	p	16
#24	ww1	n	fm	p	n	n	n	p	n	n	n	n	n	p	p	p	p	p	n	p	5
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#26	ww1	n	pc	p	n	n	n	p	n	n	n	n	p	p	n	p	n	p	n	p	4
#27	ww1	n	mb	p	p	n	n	n	n	n	n	n	p	p	n	n	n	p	p	n	13
#28	ww1	n	mb	p	p	n	p	p	n	n	n	n	n	p	p	n	n	n	n	p	15
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#30	ww1	n	mb	p	n	n	n	p	n	n	n	n	n	p	p	n	p	p	p	p	9
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#32	ww1	n	mb	p	n	n	n	n	n	n	n	n	p	p	p	p	n	n	n	n	32
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#34	ww1	n	mb	p	n	n	p	n	n	n	n	n	n	n	p	p	n	n	p	p	22
#35	ww1	n	mb	p	n	n	p	p	n	n	p	p	n	p	n	p	p	n	p	p	50
#36	ww1	n	mb	p	p	n	n	n	n	n	n	n	p	p	p	p	p	p	n	p	16
#37	ww1	n	nb	p	p	n	p	p	n	n	n	n	n	p	p	p	p	p	p	n	8
#38	ww1	n	vm	p	n	n	n	p	n	n	n	n	n	p	p	n	n	p	n	n	20
#39	ww1	n	mb	n	p	n	p	p	n	n	n	n	n	p	p	p	p	n	p	n	2
#40	ww1	n	mb	p	n	n	n	p	n	n	n	n	n	p	n	n	p	p	n	p	20
#41	ww1	n	mb	n	p	n	n	n	n	n	p	n	p	n	p	p	p	p	p	p	16
#42	ww1	n	pc	p	p	p	p	p	n	n	p	n	p	p	n	p	n	n	p	p	40
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#45	ww1	n	mb	p	p	n	n	p	n	n	p	n	n	p	n	p	n	p	p	p	40
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#93	ww2	p	mb	n	n	n	p	p	n	n	p	n	p	p	p	p	p	p	p	n	23
#94	ww2	p	fm	n	n	p	p	n	n	n	p	n	n	p	p	p	p	p	p	n	26
#95	ww2	p	fm	n	n	n	p	p	n	n	p	p	p	p	p	p	p	p	p	n	23
#96	ww2	p	pc	n	p	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	19
#97	ww2	p	mb	n	n	p	p	p	n	n	n	n	n	p	p	n	p	p	p	p	14
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#101	ww2	p	pc	n	n	p	p	p	n	n	n	n	n	p	p	p	p	p	p	n	14
#102	ww2	p	fm	p	p	n	p	p	n	n	n	n	n	p	n	p	p	p	p	n	8
#103	ww2	p	mb	n	n	n	p	p	n	n	p	n	n	n	p	p	p	p	p	n	23
#104	ww2	p	mb	n	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	42
#105	ww2	p	mb	p	n	p	p	p	n	n	p	p	n	p	p	n	p	n	p	p	39
#106	ww2	p	pc	p	n	p	p	p	n	n	p	n	n	n	p	n	p	p	p	p	39
#107	ww2	p	pc	p	n	p	p	p	n	n	n	p	p	p	p	p	p	p	p	p	27
#108	ww2	p	mb	n	n	n	p	p	n	n	p	p	n	p	p	p	p	p	p	p	6
#109	ww2	p	fm	p	n	n	p	p	n	n	n	n	n	p	p	p	n	p	p	p	49
#110	ww2	p	fm	p	n	n	n	p	n	n	p	n	p	p	p	p	p	p	p	p	28
#111	ww2	p	pc	p	p	p	p	p	n	n	n	n	n	p	p	p	p	p	p	n	27
#112	ww2	n	mb	p	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	25
#113	ww2	n	fm	p	p	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	52
#114	ww2	n	mb	n	n	p	n	p	n	n	n	n	n	p	p	p	p	p	p	p	37
#115	ww2	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	n	29
#116	ww2	n	pc	n	p	n	p	n	n	n	n	n	n	n	p	n	p	n	p	n	22
#117	ww2	n	fm	n	p	n	n	n	n	n	n	n	n	n	n	n	p	p	p	p	30
#118	ww2	n	fm	p	p	n	p	n	n	n	n	n	n	p	p	p	p	p	p	p	46
#119	ww2	n	fm	p	p	n	p	n	n	n	n	n	n	n	p	n	p	p	p	p	44
#120	ww2	n	pc	p	n	n	p	n	n	n	n	n	n	p	p	p	p	p	p	n	29
#121	ww2	n	fm	p	n	n	n	n	n	n	p	n	n	p	p	p	p	p	p	n	45
#122	ww2	n	fm	n	p	n	n	p	n	n	n	n	n	p	p	p	p	n	n	n	2

#123	ww2	n	mb	n	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#124	ww2	n	mb	p	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	5
#125	ww2	n	mb	p	n	n	n	p	n	n	n	n	n	p	n	p	n	p	p	p	25
#126	ww2	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#127	ww2	n	mb	p	n	n	p	p	n	n	n	n	n	n	p	n	p	n	p	p	22
#128	ww2	n	mb	p	n	n	n	p	n	n	n	n	n	p	n	p	p	p	p	p	25
#129	ww2	n	pc	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	n	29
#130	ww2	n	mb	p	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	n	5
#131	ww2	n	fm	p	p	n	n	p	n	n	p	n	p	p	p	p	p	p	p	p	33
#132	ww2	n	fm	p	n	n	n	p	n	n	p	n	n	p	p	p	p	p	p	p	51
#133	ww2	n	fm	p	n	n	p	n	n	n	p	n	n	n	p	n	p	p	p	p	44
#134	ww2	n	fm	p	p	n	n	n	n	n	p	n	n	p	p	p	p	p	p	n	45
#135	ww2	n	pc	p	n	n	n	n	n	n	p	n	p	p	p	p	p	p	p	n	21
#136	ww2	n	mb	n	n	n	p	n	n	n	n	n	n	p	p	p	p	p	p	p	18
#137	ww2	n	nb	n	p	n	p	n	n	n	n	n	n	p	p	p	p	p	p	n	46
#138	ww2	n	mb	p	n	n	n	n	n	n	p	p	p	p	p	p	p	p	p	n	21
#139	ww2	n	nb	p	n	n	p	n	n	n	n	n	n	p	n	p	n	p	p	p	25
#140	ww2	n	nb	n	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#141	ww2	n	mb	p	n	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	30
#142	ww2	n	nb	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	p	p	17
#143	ww2	n	mb	p	n	n	n	p	n	n	n	n	n	n	p	n	p	p	p	p	9
#144	ww2	n	mb	p	p	n	p	n	n	n	n	n	n	n	p	n	p	p	p	p	44
#145	ww2	n	fm	p	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	n	25
#146	ww2	n	fm	n	p	n	n	n	n	n	n	n	n	p	p	p	p	p	p	n	46
#147	ww2	n	fm	n	n	n	n	n	n	n	n	n	n	n	n	n	p	p	p	p	30
#148	ww2	n	fm	n	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	n	29
#149	ww2	n	mb	p	n	n	n	n	n	n	p	p	p	p	p	p	p	p	p	p	21
#150	ww2	n	mb	n	n	n	n	n	n	n	n	n	n	n	p	n	p	p	p	n	30
#151	ww2	n	fm	n	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#152	ww2	n	mb	p	p	p	n	p	n	n	p	n	n	p	n	p	n	p	p	p	40
#153	ww2	n	pc	p	n	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	30

#154	ww2	n	mb	n	p	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	10
#155	ww2	n	vm	n	p	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	10
#156	ww2	n	mb	n	p	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	10
#157	ww3	p	fm	n	n	p	p	n	n	n	n	p	p	n	p	n	p	p	p	p	3
#158	ww3	p	mb	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	42
#159	ww3	p	pc	p	p	p	p	n	n	n	p	p	p	p	p	p	n	p	p	p	1
#160	ww3	p	mb	n	n	n	n	n	n	n	p	n	n	p	p	p	p	p	p	p	26
#161	ww3	p	mb	n	n	n	n	n	n	n	n	n	n	n	p	n	p	p	p	n	30
#162	ww3	p	vm	p	n	n	n	n	n	n	n	p	p	p	p	p	n	p	p	p	42
#163	ww3	p	vm	p	p	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	19
#164	ww3	p	mb	n	p	n	p	p	n	n	n	p	n	p	p	p	p	p	p	p	43
#165	ww3	p	vm	p	p	n	n	n	n	n	n	p	n	p	p	p	n	p	p	n	12
#166	ww3	p	mb	p	n	p	n	p	n	n	n	n	n	p	p	n	p	p	p	p	9
#167	ww3	p	mb	p	n	p	p	n	n	n	n	n	n	p	p	p	p	p	p	p	18
#168	ww3	p	mb	p	p	n	p	p	n	n	n	n	p	p	p	p	p	p	p	p	19
#169	ww3	p	fm	p	n	p	p	p	n	n	n	p	n	p	p	p	p	p	p	p	27
#170	ww3	p	mb	p	n	n	n	p	n	n	n	p	p	p	p	p	p	p	p	p	42
#171	ww3	p	mb	p	n	n	n	p	n	n	n	n	n	p	p	n	p	p	p	p	9
#172	ww3	p	mb	p	n	n	n	n	n	n	p	p	n	p	p	p	p	p	p	p	17
#173	ww3	p	mb	p	n	n	n	n	n	n	n	p	n	n	p	n	p	p	p	n	30
#174	ww3	p	mb	p	n	p	p	p	n	n	n	n	n	p	p	p	p	p	p	p	27
#175	ww3	p	pc	p	p	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	19
#176	ww3	p	fm	n	p	n	p	p	n	n	p	n	p	p	p	p	p	p	p	p	23
#177	ww3	p	fm	p	n	p	p	p	n	n	n	p	n	p	p	p	p	p	p	p	27
#178	ww3	p	mb	p	n	p	n	p	n	n	n	n	n	p	p	n	p	p	p	p	9
#179	ww3	p	mb	n	n	p	p	p	n	n	n	n	n	p	p	n	p	p	p	p	14
#180	ww3	p	pc	p	p	n	n	p	n	n	p	n	p	p	p	p	p	p	p	p	28
#181	ww3	p	pc	p	p	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#182	ww3	p	fm	n	p	n	n	p	n	n	n	p	n	p	p	p	p	p	p	p	43
#183	ww3	p	pc	p	p	p	p	p	n	n	n	n	p	p	p	p	p	p	p	p	19
#184	ww3	p	mb	p	p	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	19

#185	ww3	p	pc	p	p	n	p	p	n	n	n	p	n	p	p	n	p	p	p	p	12
#186	ww3	p	fm	n	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#187	ww3	p	fm	n	n	n	p	p	n	n	n	p	n	p	p	p	p	p	p	p	6
#188	ww3	p	wa	n	p	n	p	p	n	n	n	n	p	p	n	p	p	p	p	n	8
#189	ww3	p	nb	p	n	p	p	p	n	n	n	p	n	p	p	n	p	n	p	p	39
#190	ww3	p	mb	n	n	p	n	p	n	n	p	n	p	n	p	n	p	p	p	p	39
#191	ww3	p	nb	n	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	47
#192	ww3	p	vm	p	p	n	p	p	n	n	n	p	n	p	n	p	p	p	p	p	12
#193	ww3	p	fm	p	n	p	p	p	n	n	n	p	n	p	n	p	p	p	p	n	27
#194	ww3	p	pc	n	n	p	p	p	n	n	n	n	n	p	n	p	p	p	p	p	47
#195	ww3	p	mb	n	p	p	p	p	n	n	n	n	n	p	p	p	p	p	p	p	19
#196	ww3	p	mb	n	p	n	p	p	n	n	p	n	p	p	p	p	p	p	p	p	23
#197	ww3	p	mb	p	p	p	n	p	n	n	n	p	n	p	p	p	p	p	p	p	27
#198	ww3	p	nb	p	p	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	19
#199	ww3	p	mb	n	p	n	n	n	n	n	p	n	p	p	p	p	p	p	p	p	23
#200	ww3	p	pc	n	n	n	n	n	n	n	n	n	p	p	p	p	n	p	p	p	18
#201	ww3	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#202	ww3	n	mb	p	n	n	n	n	n	n	p	n	n	p	p	p	p	p	p	p	45
#203	ww3	n	pc	p	p	n	n	n	n	n	p	n	p	p	p	p	p	p	p	p	33
#204	ww3	n	nb	p	p	p	n	p	n	n	n	p	n	p	p	n	n	p	p	p	13
#205	ww3	n	vm	P	n	n	p	p	n	n	p	p	n	p	p	p	p	p	p	p	50
#206	ww3	n	nb	p	n	n	n	p	n	n	p	n	p	p	p	p	p	p	p	p	51
#207	ww3	n	pc	p	n	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	5
#208	ww3	n	mb	p	p	p	p	n	n	n	n	n	n	n	p	n	p	p	p	p	44
#209	ww3	n	mb	n	p	n	n	n	n	n	n	n	p	p	p	p	p	p	p	p	16
#210	ww3	n	nb	p	n	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	29
#211	ww3	n	mb	p	n	p	p	p	n	n	p	p	p	p	p	p	p	n	p	n	31
#212	ww3	n	mb	p	p	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	52
#213	ww3	n	nb	p	p	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	52
#214	ww3	n	pc	p	n	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	30
#215	ww3	n	vm	p	p	n	n	p	n	n	n	n	n	p	p	n	n	p	p	p	13

#216	ww3	n	mb	p	n	n	n	p	n	n	p	p	p	p	p	p	p	p	p	p	21
#217	ww3	n	nb	p	p	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	52
#218	ww3	n	nb	p	p	n	n	n	n	n	n	n	p	p	p	n	n	p	p	p	13
#219	ww3	n	nb	p	p	n	n	p	n	n	p	p	p	p	p	p	p	p	p	p	33
#220	ww3	n	vm	p	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	25
#221	ww3	n	nb	p	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	5
#222	ww3	n	mb	p	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	5
#223	ww3	n	nb	p	p	n	n	n	n	n	p	n	n	p	p	p	p	p	p	p	45
#224	ww3	n	nb	p	p	n	n	p	n	n	p	n	n	p	p	n	n	p	p	p	13
#225	ww3	n	fm	p	n	n	n	n	n	n	n	p	n	p	p	p	p	p	p	p	17
#226	ww3	n	fm	p	n	n	n	p	n	n	n	n	p	p	p	p	p	p	p	p	38
#227	ww3	n	fm	p	p	n	n	p	n	n	p	p	p	p	p	p	p	p	p	p	33
#228	ww3	n	fm	p	p	p	p	p	n	n	n	p	n	p	p	p	p	p	p	p	27
#229	ww3	n	wa	p	p	n	p	p	n	n	n	n	n	p	n	p	p	p	p	n	8
#230	ww3	n	fm	p	p	n	p	p	n	n	p	n	p	p	p	p	p	n	p	p	33
#231	ww3	n	fm	p	n	n	n	p	n	n	p	n	n	p	n	p	n	n	p	n	48
#232	ww3	n	fm	p	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	5
#233	ww3	n	fm	p	n	n	n	p	n	n	n	n	n	p	n	p	n	p	p	p	25
#234	ww3	n	pc	p	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	5
#235	ww3	n	mb	p	n	n	n	n	n	n	p	n	n	p	n	n	p	p	p	p	25
#236	ww3	n	mb	n	n	p	n	p	n	n	n	n	n	p	p	p	n	p	p	p	37
#237	ww3	n	pc	p	p	p	p	p	n	n	n	n	n	p	p	p	p	p	n	p	27
#238	ww3	n	vm	n	n	p	p	p	n	n	n	n	n	p	n	p	p	p	p	p	47
#239	ww3	n	mb	n	n	p	n	p	n	n	p	n	n	p	p	p	p	p	p	p	37
#240	ww3	n	mb	n	n	p	n	n	n	n	p	n	p	p	p	p	p	p	p	p	51
#241	ww3	n	mb	n	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#242	ww3	n	mb	n	n	n	n	n	n	n	p	n	p	p	p	p	p	p	p	p	51
#243	ww3	n	mb	p	p	n	n	p	n	n	n	p	n	p	n	p	p	p	p	p	52
#244	ww3	n	nb	n	p	n	n	p	n	n	n	p	n	p	p	p	p	p	p	p	43
#245	ww3	n	nb	n	n	n	n	n	n	n	p	n	p	p	p	p	p	p	p	p	51
#246	ww3	n	mb	n	n	n	n	p	n	n	p	n	p	p	p	p	p	p	p	p	51

#247	ww3	n	mb	p	n	n	n	p	n	n	n	n	n	p	n	p	p	p	p	p	25
#248	ww3	n	mb	n	p	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	10
#249	ww3	n	mb	n	n	n	n	p	n	n	n	n	n	p	n	p	p	p	p	p	25
#250	ww3	n	mb	n	n	p	n	p	n	n	n	p	n	p	p	p	p	p	p	p	37
#251	ww3	n	mb	n	n	n	n	n	n	n	p	p	p	p	p	p	p	p	n	n	54
#252	ww3	n	mb	n	p	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	10
#253	ss1	n	nb	n	n	n	n	n	n	n	n	p	p	n	p	n	p	p	p	p	3
#254	ss1	n	nb	p	n	n	n	n	n	n	p	p	n	p	p	p	p	p	p	p	17
#255	ss1	n	pc	p	n	n	n	n	n	n	p	n	p	p	p	p	p	p	p	p	21
#256	ss1	n	mb	p	n	n	n	p	n	n	n	n	p	p	p	n	p	p	p	p	24
#257	ss1	n	pc	n	n	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	30
#258	ss1	n	nb	p	n	n	n	p	n	n	n	n	n	p	p	p	n	p	p	p	29
#259	ss1	n	nb	n	n	n	n	p	n	n	n	n	n	n	p	n	p	p	p	p	30
#260	ss1	n	nb	p	n	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	5
#261	ss1	n	nb	p	n	n	n	p	n	n	n	n	n	p	n	p	n	p	p	p	25
#262	ss1	n	pc	p	n	n	n	n	n	n	n	n	n	n	n	n	p	p	p	p	30
#263	ss1	n	nb	p	n	n	n	p	n	n	n	n	p	p	p	p	p	p	p	p	38
#264	ss1	n	nb	n	n	n	n	p	n	n	p	n	p	p	p	p	p	p	p	p	51
#265	ss1	n	pc	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#266	ss1	n	nb	p	n	n	p	p	n	n	p	n	p	p	p	p	n	p	p	p	4
#267	ss1	n	mb	n	p	n	n	p	n	n	n	n	p	p	p	n	p	p	p	p	43
#268	ss1	n	pc	p	n	n	n	p	n	n	n	n	p	p	p	p	p	p	p	p	38
#269	ss1	n	nb	p	n	n	n	p	n	n	n	n	n	n	p	n	p	p	p	p	9
#270	ss1	n	mb	p	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	p	25
#271	ss1	p	mb	n	n	n	p	n	n	n	n	n	n	p	n	p	p	p	p	p	47
#272	ss1	n	nb	p	n	n	n	n	n	n	p	n	n	p	p	p	p	p	p	p	45
#273	ss1	n	nb	p	n	n	n	p	n	n	n	n	n	p	n	p	p	p	p	p	25
#274	ss1	n	nb	p	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#275	ss1	n	nb	p	n	n	p	n	n	n	n	n	n	p	p	n	p	p	p	p	44
#276	ss1	n	nb	p	n	n	n	n	n	n	p	n	p	p	p	p	p	p	p	p	21
#277	ss1	p	nb	n	n	n	p	n	n	n	n	n	p	n	p	n	p	p	p	p	3

#278	ss1	n	nb	p	n	n	n	n	n	n	n	n	p	p	p	p	p	p	n	38
#279	ss1	p	nb	n	n	n	p	n	n	n	p	n	n	p	p	p	p	p	p	p	26
#280	ss1	p	nb	n	n	p	p	n	n	n	p	n	n	p	p	p	p	p	p	p	26
#281	ss1	n	nb	p	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#282	ss1	n	fm	p	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	p	24
#283	ss1	p	mb	p	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	n	18
#284	ss1	n	mb	p	n	n	n	n	n	n	n	n	n	n	p	n	p	n	p	n	22
#285	ss1	n	pc	n	n	n	p	n	n	n	n	n	p	p	p	p	p	p	n	n	54
#286	ss1	n	fm	p	n	n	p	n	n	n	n	n	p	p	p	n	p	p	p	p	44
#287	ss1	n	fm	n	n	n	p	n	n	n	p	n	p	n	p	n	p	p	p	p	44
#288	ss1	p	fm	p	n	n	p	n	n	n	n	n	p	p	p	p	p	p	p	p	18
#289	ss1	p	fm	p	n	n	p	n	n	n	p	n	p	p	p	p	p	p	p	p	18
#290	ss1	p	fm	p	n	p	p	n	n	n	n	n	p	p	p	p	p	p	p	p	18
#291	ss1	p	fm	p	n	n	n	n	n	n	p	n	n	p	p	p	p	p	p	p	42
#292	ss1	n	pc	p	n	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	30
#293	ss1	n	mb	p	n	n	n	n	n	n	n	n	p	p	p	p	n	p	p	p	4
#294	ss1	n	fm	p	n	n	n	p	n	n	n	n	p	p	p	p	p	p	p	p	38
#295	ss1	n	fm	p	n	n	n	p	n	n	n	n	p	p	p	p	p	p	p	p	38
#296	ss1	n	fm	p	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	p	38
#297	ss1	n	fm	p	p	n	n	n	n	n	n	n	p	p	p	p	p	p	p	p	16
#298	ss1	n	mb	p	n	n	p	n	n	n	p	n	p	p	p	p	p	p	p	p	21
#299	ss1	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	n	29
#300	ss1	n	mb	p	n	n	n	p	n	n	n	n	p	p	p	p	p	p	p	p	38
#301	ss1	p	mb	p	n	n	p	n	n	n	n	n	p	p	p	p	n	p	p	p	18
#302	ss1	n	pc	p	n	n	n	n	n	n	p	n	p	p	p	p	n	n	p	p	21
#303	ss1	n	pc	p	n	p	p	n	n	n	n	n	p	p	p	n	n	p	p	p	44
#304	ss1	n	fm	p	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	p	24
#305	ss1	n	vm	p	n	n	n	n	n	n	n	p	n	p	p	n	p	p	n	p	17
#306	ss1	p	vm	p	n	n	p	n	n	n	n	p	n	p	p	n	p	p	p	p	12
#307	ss1	n	fm	p	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#308	ss1	n	pc	p	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	p	24

#309	ss1	n	mb	p	n	n	n	n	n	n	n	p	p	p	p	p	n	p	p	n	32
#310	ss1	p	mb	p	n	n	p	p	n	n	n	n	p	p	p	p	n	p	p	n	49
#311	ss1	n	fm	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#312	ss1	n	nb	p	p	n	p	n	n	n	n	n	n	p	p	p	p	p	p	n	46
#313	ss1	n	nb	p	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	p	24
#314	ss1	n	nb	p	n	n	n	p	n	n	n	n	p	p	p	p	p	p	p	p	38
#315	ss1	n	nb	p	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	p	24
#316	ss1	n	nb	p	n	n	n	n	n	n	n	n	n	n	p	n	p	p	p	p	30
#317	ss1	n	nb	p	n	n	n	p	n	n	n	n	p	p	p	n	p	p	p	p	24
#318	ss1	n	nb	p	n	n	n	p	n	n	n	n	p	p	p	n	n	p	p	p	24
#319	ss1	n	nb	p	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	p	24
#320	ss1	n	nb	n	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	p	24
#321	ss1	n	nb	p	n	n	n	p	n	n	n	n	p	p	p	n	p	p	p	p	24
#322	ss1	p	nb	n	n	n	n	n	n	n	n	n	p	p	p	n	p	p	n	p	3
#323	ss1	n	nb	n	p	n	p	p	n	n	n	n	n	p	p	n	p	p	p	p	43
#324	ss1	n	pc	p	n	n	n	n	n	n	n	n	n	p	p	p	n	p	p	p	29
#325	ss1	p	mb	p	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#326	ss1	p	mb	n	p	n	n	n	n	n	p	n	n	p	p	n	p	p	p	p	36
#327	ss1	n	fm	p	n	p	p	p	n	n	n	n	n	p	p	n	p	p	p	p	14
#328	ss1	n	fm	p	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	p	38
#329	ss1	n	nb	p	n	n	n	n	n	n	n	p	p	p	p	n	p	p	p	p	24
#330	ss1	n	mb	p	p	n	n	p	n	n	n	n	n	p	p	p	p	p	n	p	5
#331	ss1	n	nb	p	n	n	n	n	n	n	n	p	n	p	p	n	p	n	p	p	17
#332	ss1	p	mb	p	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#333	ss1	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#334	ss1	n	mb	p	n	n	n	n	n	n	p	p	p	p	p	p	p	n	p	n	21
#335	ss1	n	fm	p	n	n	n	p	n	n	n	n	p	p	p	p	n	p	p	p	4
#336	ss1	n	mb	n	n	p	p	p	n	n	n	n	n	p	p	n	p	p	p	p	14
#337	ss1	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	n	p	p	p	p	53
#338	ss1	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	p	p	29
#339	ss1	n	mb	p	n	n	n	n	n	n	n	n	n	p	p	p	p	p	n	p	5

#340	ss1	n	mb	p	p	n	p	n	n	n	n	n	p	p	p	p	p	n	p	n	46
#341	ss1	n	nb	p	n	n	n	n	n	n	n	n	p	p	n	n	p	p	p	p	24
#342	ss1	n	mb	n	n	p	p	p	n	n	n	n	n	p	p	p	n	p	p	p	37
#343	ss1	n	fm	p	n	n	n	p	n	n	n	n	n	n	n	n	p	n	n	n	34
#344	ss1	n	nb	p	n	n	n	n	n	n	p	n	p	p	p	p	p	n	p	p	21
#345	ss1	n	mb	n	p	n	n	n	n	n	p	n	n	n	p	n	n	p	n	n	15
#346	ss1	n	nb	n	p	n	p	p	n	n	n	n	n	n	n	n	p	p	n	n	34
#347	ss1	n	fm	n	n	n	n	p	n	n	p	n	n	n	n	n	p	n	n	n	34
#348	ss1	p	nb	p	n	n	n	n	n	n	n	n	n	p	p	p	n	n	p	n	32
#349	ss1	n	vm	p	n	n	n	p	n	n	n	n	n	p	n	p	p	n	n	n	20
#350	ss1	n	nb	p	n	n	n	p	n	n	p	p	n	p	p	p	p	n	n	n	35
#351	ss1	p	nb	p	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	34
#352	ss1	n	mb	p	n	n	p	p	n	n	p	n	p	p	p	p	p	n	n	n	35
#353	ss1	n	nb	p	n	n	n	p	n	n	n	n	p	p	n	p	p	n	n	p	20
#354	ss1	n	mb	n	n	p	p	p	n	n	p	n	n	n	n	n	p	p	n	p	7
#355	ss1	n	mb	n	n	p	p	p	n	n	n	n	n	n	p	n	n	n	n	n	34

Ww-wastewater, ss-soil sample, Bc-biofilm class, pc-physical cohesive, fm-floccular mass, mb-meniscus biofilm, nb- no biofilm, vm-viscous mass, wa-waxy aggregates, p-positive, n-negative, Oxid, oxidase, Cat, catalase, Surf, surfactant, Swim, swimming, Mucd, mucoidal, Cuac, culture acidity, Nacl, Sodium chloride tolrance, Sider, sideropore, Swar, swarming, Twit, twitching, L sec, lipase secretion, Gel, gelatinase activity, Sug, sugar utilization, Prot, protease secretion, Acpr, acid production in the presence of maltose, Hg, mercury, Tet, tetracycline, Kan, kanamycin, Clst, cluster.

Appendix A2

Additional information for Chapter 4

Table A2.1 Mean liquid surface tension activities of 85 pseudomonad isolates using tensiometry

Strain	Mean ± SE	Strain	Mean ± SE
Strain 83	24.817 ± 0.1	Strain 193	32.119 ± 0.9
Strain 69	24.455 ± 0.1	Strain 81	31.896 ± 1.5
Strain 101	24.857 ± 0.0	Strain 280	33.489 ± 0.2
Strain 303	25.080 ± 0.0	Strain 167	35.068 ± 1.4
Strain 190	25.104 ± 0.1	Strain 42	36.675 ± 0.7
Strain 152	25.142 ± 0.0	Strain 228	38.809 ± 0.7
Strain 178	25.240 ± 0.4	Strain 67	42.136 ± 0.3
Strain 6	25.244 ± 0.2	Strain 312	46.645 ± 1.0
Strain 1	25.297 ± 0.1	Strain 177	47.203 ± 1.2
Strain 65	25.324 ± 0.2	Strain 310	47.935 ± 0.6
Strain 211	25.329 ± 0.1	Strain 250	48.560 ± 0.5
Strain 194	25.405 ± 0.1	Strain 238	48.565 ± 0.7
Strain 79	25.469 ± 0.1	Strain 92	48.945 ± 1.0
Strain 97	25.721 ± 0.1	Strain 47	50.117 ± 0.1
Strain 179	25.478 ± 0.1	Strain 75	49.054 ± 1.4
Strain 236	26.351 ± 0.1	Strain 14	50.506 ± 0.2
Strain 2	25.948 ± 0.1	Strain 157	50.334 ± 0.7
Strain 197	26.397 ± 0.0	Strain 174	50.983 ± 0.2
Strain 88	26.532 ± 0.1	Strain 290	49.783 ± 1.8
Strain 169	26.404 ± 0.1	Strain 204	51.101 ± 0.5
Strain 239	26.553 ± 0.1	Strain 58	51.403 ± 0.2
Strain 86	25.528 ± 0.1	Strain 114	49.985 ± 2.1
Strain 175	26.695 ± 0.2	Strain 304	52.418 ± 0.4
Strain 336	26.723 ± 0.1	Strain 305	52.653 ± 0.3
Strain 355	26.759 ± 0.1	Strain 17	52.668 ± 0.4
Strain 166	26.700 ± 0.2	Strain 315	52.613 ± 0.5
Strain 335	26.705 ± 0.1	Strain 309	52.634 ± 0.5
Strain 5	30.310 ± 0.1	KB media	52.777 ± 0.5
Strain 107	26.904 ± 0.1	Strain 46	52.915 ± 0.4
Strain 105	26.798 ± 0.1	Strain 237	53.160 ± 0.9
Strain 354	27.325 ± 0.0	Strain 313	54.122 ± 0.5
Strain 111	26.912 ± 0.2	Strain 240	53.471 ± 1.3
Strain 94	27.177 ± 0.2	Strain 61	53.608 ± 1.6
Strain 106	28.448 ± 0.1	Strain 307	55.030 ± 0.8
Strain 195	27.824 ± 0.4	Strain 208	54.939 ± 0.9
Strain 327	27.890 ± 0.2	Strain 73	54.963 ± 1.0
Strain 85	28.874 ± 0.1	Strain 51	56.605 ± 0.3
Strain 9	28.553 ± 0.0	Strain 64	55.438 ± 1.7
Strain 8	28.566 ± 0.2	Strain 159	56.495 ± 1.9
Strain 183	29.155 ± 0.2	Strain 314	58.151 ± 0.3
Strain 63	28.914 ± 0.1	Strain 308	56.919 ± 1.8
Strain 342	29.115 ± 0.9	Strain 306	57.876 ± 2.1
Strain 189	29.889 ± 0.2	Strain 311	59.180 ± 0.8

Table A2.2 Mean liquid surface tension activities of 355 pseudomonad isolates by tensiometry

Strain	Mean ± SE	Strain	Mean ± SE	Strain	Mean ± SE
Strain 69	24.455 ± 0.1	Strain 354	27.325 ± 0.0	Strain 204	51.101 ± 0.5
Strain 83	24.817 ± 0.1	Strain 195	27.824 ± 0.4	Strain 290	49.783 ± 1.8
Strain 101	24.857 ± 0.0	Strain 327	27.890 ± 0.2	Strain 58	51.403 ± 0.2
Strain 303	25.080 ± 0.0	Strain 106	28.448 ± 0.1	Strain 304	52.418 ± 0.4
Strain 190	25.104 ± 0.1	Strain 9	28.553 ± 0.0	Strain 315	52.613 ± 0.5
Strain 152	25.142 ± 0.0	Strain 8	28.566 ± 0.2	Strain 309	52.634 ± 0.5
Strain 178	25.240 ± 0.4	Strain 85	28.874 ± 0.1	Strain 305	52.653 ± 0.3
Strain 6	25.244 ± 0.2	Strain 63	28.914 ± 0.1	Strain 17	52.668 ± 0.4
Strain 1	25.297 ± 0.1	Strain 342	29.115 ± 0.9	Strain 46	52.915 ± 0.4
Strain 65	25.324 ± 0.2	Strain 183	29.155 ± 0.2	Strain 237	53.160 ± 0.9
Strain 211	25.329 ± 0.1	Strain 189	29.889 ± 0.2	Strain 240	53.471 ± 1.3
Strain 194	25.405 ± 0.1	Strain 5	30.310 ± 0.1	Strain 61	53.608 ± 1.6
Strain 79	25.469 ± 0.1	Strain 81	31.896 ± 1.5	Strain 313	54.122 ± 0.5
Strain 179	25.478 ± 0.1	Strain 193	32.119 ± 0.9	Strain 208	54.939 ± 0.9
Strain 86	25.528 ± 0.1	Strain 280	33.489 ± 0.2	Strain 73	54.963 ± 1.0
Strain 97	25.721 ± 0.1	Strain 167	35.068 ± 1.4	Strain 307	55.030 ± 0.8
Strain 2	25.948 ± 0.1	Strain 42	36.675 ± 0.7	Strain 64	55.438 ± 1.7
Strain 236	26.351 ± 0.1	Strain 228	38.809 ± 0.7	Strain 159	56.495 ± 1.9
Strain 197	26.397 ± 0.0	Strain 67	42.136 ± 0.3	Strain 51	56.605 ± 0.3
Strain 169	26.404 ± 0.1	Strain 312	46.645 ± 1.0	Strain 308	56.919 ± 1.8
Strain 88	26.532 ± 0.1	Strain 177	47.203 ± 1.2	Strain 306	57.876 ± 2.1
Strain 239	26.553 ± 0.1	Strain 310	47.935 ± 0.6	Strain 314	58.151 ± 0.3
Strain 175	26.695 ± 0.2	Strain 250	48.560 ± 0.5	Strain 311	59.180 ± 0.8
Strain 166	26.700 ± 0.2	Strain 238	48.565 ± 0.7	KB media	52.777 ± 0.5
Strain 335	26.705 ± 0.1	Strain 92	48.945 ± 1.0	ST of Water	73.156 ± 0.1
Strain 336	26.723 ± 0.1	Strain 75	49.054 ± 1.4	Used Lubri. Oil	29.365 ± 0.4
Strain 355	26.759 ± 0.1	Strain 114	49.985 ± 2.1	Strain SBW 25	26.519 ± 0.0
Strain 107	26.904 ± 0.1	Strain 47	50.117 ± 0.1	Strain Mass A	58.359 ± 0.2
Strain 105	26.798 ± 0.1	Strain 157	50.334 ± 0.7	Strain RSS 101	25.262 ± 0.1
Strain 111	26.912 ± 0.2	Strain 14	50.506 ± 0.2	Hexadecane	65.193 ± 0.2
Strain 94	27.177 ± 0.2	Strain 174	50.983 ± 0.2	Strain visc A	57.088 ± 0.3

Table A2.3 Posthoc multiple comparison test and the resulting homogeneous classes

		Subset																						
Strain	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Strain 69	4	24.45475																						
Strain 83	4	24.81700	24.81700																					
Strain 101	4	24.85700	24.85700	24.85700																				
Strain 303	4	25.08000	25.08000	25.08000																				
Strain 190	4	25.10350	25.10350	25.10350																				
Strain 152	4	25.14150	25.14150	25.14150																				
Strain 178	4	25.23950	25.23950	25.23950																				
Strain 6	4	25.24350	25.24350	25.24350																				
Strain 1	4	25.29700	25.29700	25.29700																				
Strain 65	4	25.32350	25.32350	25.32350																				
Strain 211	4	25.32900	25.32900	25.32900																				
Strain 194	4	25.40450	25.40450	25.40450																				
Strain 79	4	25.46850	25.46850	25.46850																				
Strain 179	8	25.47825	25.47825	25.47825																				
Strain 86	4	25.52750	25.52750	25.52750																				
Strain 97	4	25.72050	25.72050	25.72050	25.72050																			
Strain 2	4	25.94775	25.94775	25.94775	25.94775																			
Strain 236	4	26.35125	26.35125	26.35125	26.35125	26.35125																		
Strain 197	4	26.39700	26.39700	26.39700	26.39700	26.39700																		
Strain 169	4	26.40350	26.40350	26.40350	26.40350	26.40350																		
Strain 88	4	26.53150	26.53150	26.53150	26.53150	26.53150																		
Strain 239	4	26.55325	26.55325	26.55325	26.55325	26.55325																		
Strain 175	4	26.69450	26.69450	26.69450	26.69450	26.69450																		
Strain 166	4	26.70025	26.70025	26.70025	26.70025	26.70025																		
Strain 335	4	26.70525	26.70525	26.70525	26.70525	26.70525																		
Strain 336	4	26.72275	26.72275	26.72275	26.72275	26.72275																		
Strain 355	4	26.75925	26.75925	26.75925	26.75925	26.75925																		
Strain 107	6	26.83767	26.83767	26.83767	26.83767	26.83767																		
Strain 105	2	26.89050	26.89050	26.89050	26.89050	26.89050																		
Strain 111	4	26.91175	26.91175	26.91175	26.91175	26.91175																		
Strain 94	4	27.17675	27.17675	27.17675	27.17675	27.17675																		
Strain 354	4	27.32500	27.32500	27.32500	27.32500	27.32500																		
Strain 195	4	27.82400	27.82400	27.82400	27.82400	27.82400	27.82400																	
Strain 327	4	27.89000	27.89000	27.89000	27.89000	27.89000	27.89000																	
Strain 106	4	28.44750	28.44750	28.44750	28.44750	28.44750	28.44750																	
Strain 9	4	28.55275	28.55275	28.55275	28.55275	28.55275	28.55275																	
Strain 8	4	28.56600	28.56600	28.56600	28.56600	28.56600	28.56600																	
Strain 85	4		28.87350	28.87350	28.87350	28.87350	28.87350																	
Strain 63	4		28.91400	28.91400	28.91400	28.91400	28.91400																	
Strain 342	4		29.11525	29.11525	29.11525	29.11525	29.11525																	
Strain 183	4			29.15525	29.15525	29.15525	29.15525																	
Strain 189	4				29.88850	29.88850	29.88850	29.88850																
Strain 5	4					30.31000	30.31000	30.31000																
Strain 81	4						31.89600	31.89600	31.89600															
Strain 193	3						32.11867	32.11867	32.11867															
Strain 280	4							33.48925	33.48925	33.48925														
Strain 167	4								35.06775	35.06775	35.06775													
Strain 42	4									36.67500	36.67500													
Strain 228	3										38.80900	38.80900												
Strain 67	4											42.13600												
Strain 312	3												46.64500											
Strain 177	4												47.20300	47.20300										
Strain 310	4												47.93500	47.93500										
Strain 250	4												48.56025	48.56025	48.56025									
Strain 238	2												48.56500	48.56500	48.56500									
Strain 92	4												48.94500	48.94500	48.94500	48.94500								
Strain 75	4												49.05350	49.05350	49.05350	49.05350								
Strain 114	4												49.98525	49.98525	49.98525	49.98525	49.98525							
Strain 47	4												50.11700	50.11700	50.11700	50.11700	50.11700							
Strain 157	4												50.33400	50.33400	50.33400	50.33400	50.33400							
Strain 14	4												50.50575	50.50575	50.50575	50.50575	50.50575							
Strain 174	4													50.98250	50.98250	50.98250	50.98250	50.98250						
Strain 204	4													51.10100	51.10100	51.10100	51.10100	51.10100						
Strain 290	3													51.34100	51.34100	51.34100	51.34100	51.34100	51.34100					
Strain 58	4													51.40325	51.40325	51.40325	51.40325	51.40325	51.40325					
Strain 304	4														52.41825	52.41825	52.41825	52.41825	52.41825	52.41825				
Strain 315	4														52.61275	52.61275	52.61275	52.61275	52.61275	52.61275	52.61275			
Strain 309	4														52.63350	52.63350	52.63350	52.63350	52.63350	52.63350	52.63350			
Strain 305	4														52.65325	52.65325	52.65325	52.65325	52.65325	52.65325	52.65325			
Strain 17	4														52.66825	52.66825	52.66825	52.66825	52.66825	52.66825	52.66825			
Strain 46	4															52.91475	52.91475	52.91475	52.91475	52.91475	52.91475			
Strain 237	3															53.15967	53.15967	53.15967	53.15967	53.15967	53.15967			
Strain 240	4																53.47100	53.47100	53.47100	53.47100	53.47100			
Strain 61	4																53.60775	53.60775	53.60775	53.60775	53.60775	53.60775		
Strain 313	4																54.12200	54.12200	54.12200	54.12200	54.12200	54.12200	54.12200	
Strain 208	4																	54.93850	54.93850	54.93850	54.93850	54.93850	54.93850	54.93850
Strain 73	4																	54.96300	54.96300	54.96300	54.96300	54.96300	54.96300	54.96300
Strain 307	4																	55.02975	55.02975	55.02975	55.02975	55.02975	55.02975	55.02975
Strain 64	4																		55.43775	55.43775	55.43775	55.43775	55.43775	55.43775
Strain 159	3																			56.49467	56.49467	56.49467	56.49467	56.49467
Strain 51	4																			56.60525	56.60525	56.60525	56.60525	56.60525
Strain 308	4																				56.91875	56.91875	56.91875	56.91875
Strain 306	4																					57.87575	57.87575	57.87575
Strain 314	4																						58.15100	58.15100
Strain 311	4																							59.17975
Sig.		.097	.052	.052	.081	.153	.053	.375	.740	.729	.273	.609	.201	.073	.098	.069	.089	.118	.101	.076	.051	.058	.125	.063

From this table the strains separated very well into two homogeneous groups (significantly different one of each other). The separation occurs between strain 67 and strain 312.

Appendix A3

Additional information for Chapter 5

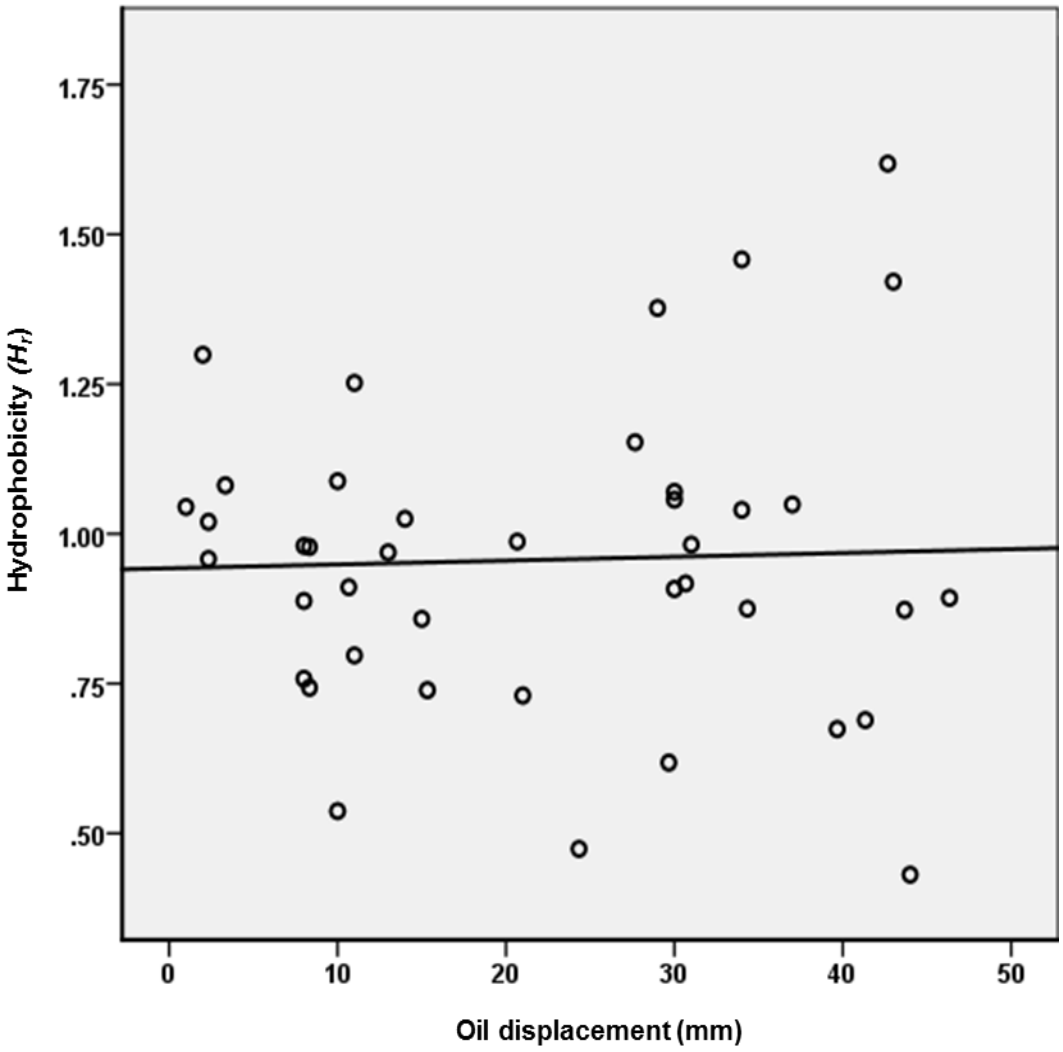

Figure A3.1 Scatter plots of correlations between hydrophobicity and oil displacement. Shown is the scatter plot of the correlations between hydrophobicity and oil displacement with a significant interaction (P = 0.357) and a weak relationship between the two variables (r = 0.429).

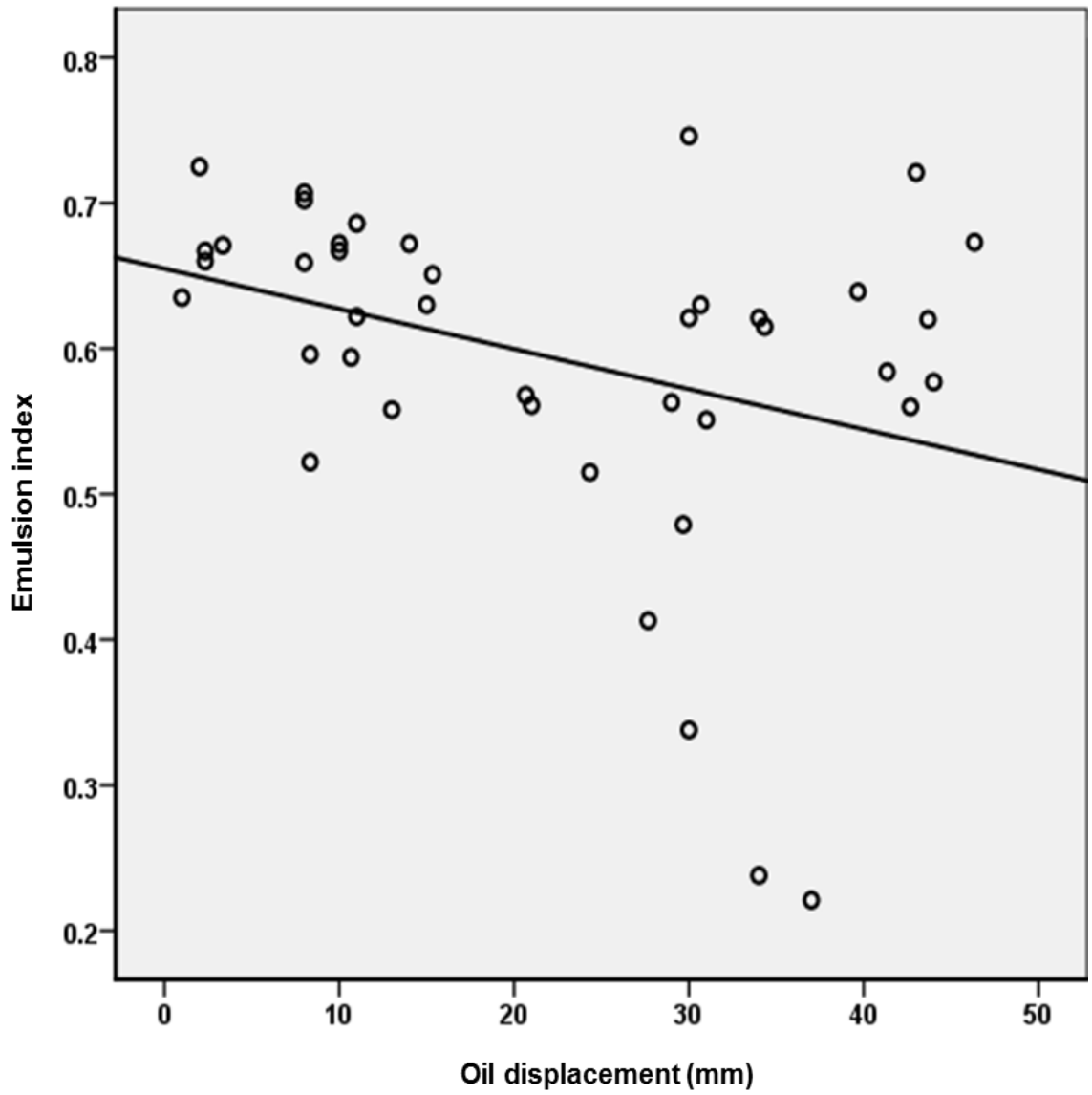

Figure A3.2 Scatter plots of correlations between mean emulsion index and oil displacement. Shown is the relationship between emulsion index and oil displacement with a highly significant interaction (P =0.031) a weak or moderate linear correlation (r = 0.338).

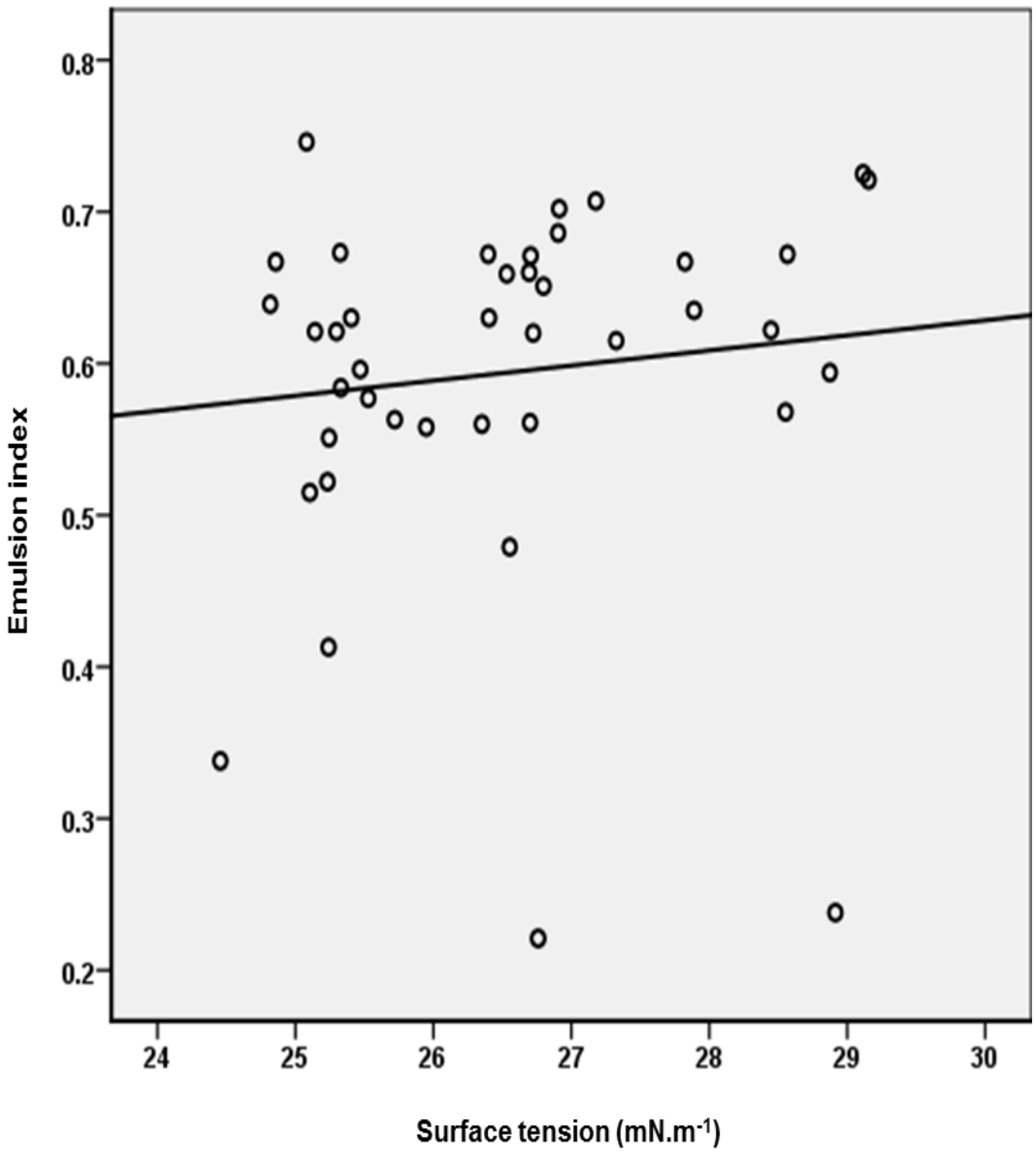

Figure A3.3 Scatter plots of correlations between emulsion index and surface tension. Shown is the scatter plot of emulsion index and surface tension of isolates with a non-significant interaction amongst the two systems (*P* = 0.476) very weak linear relationship (r = 0.114).

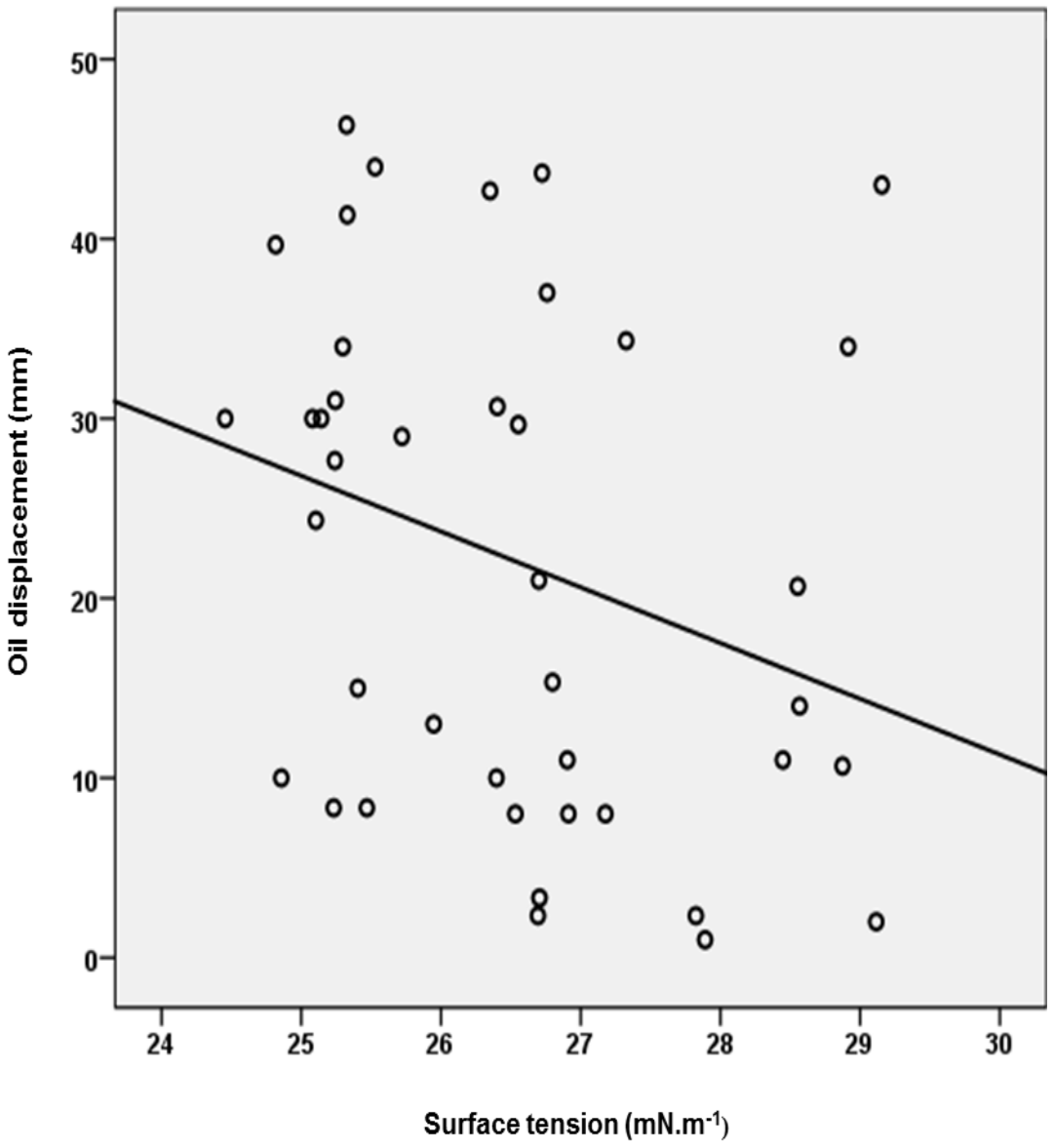

Figure A3.4 Scatter plots of correlations between oil displacement and surface tension. Shown is the scatter plot of the mean oil displacement and surface tension with a significant interaction amongst the two systems (*P* = 0.064) with a weak linear relationship (r = 0.292).

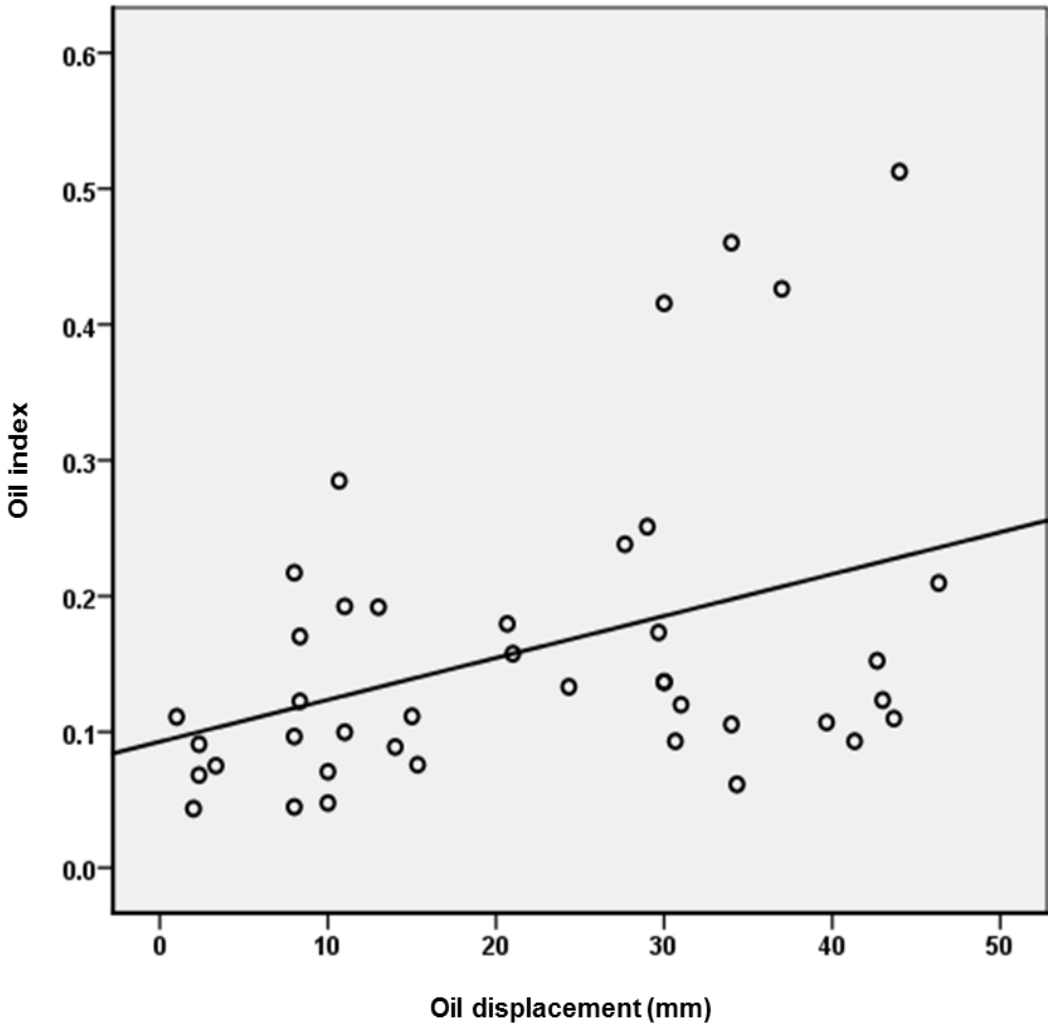

Figure A3.5 Scatter plots of correlations between mean oil index and oil displacement. Shown is the scatter plot of the oil index and oil displacement with a significant interaction between the two systems (*P* = 0.013) and a weak correlations (r = 0.387).

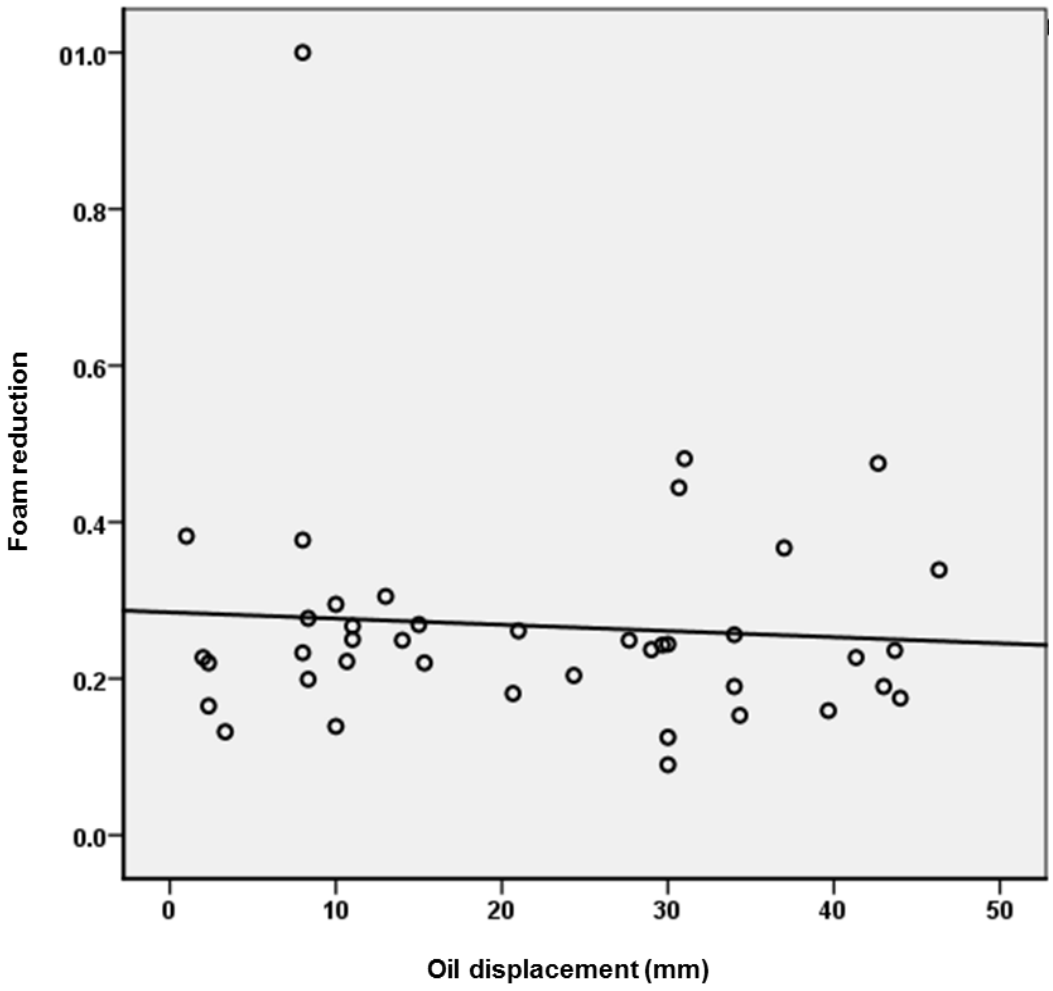

Figure A3.6 Scatter plots of correlations between foam reduction and oil displacement. Shown is the scatter plot of foam reduction and oil displacement with a non-significant interaction between the two systems (*P* = 0.636) with a very weak linear correlations (r = 0.429).

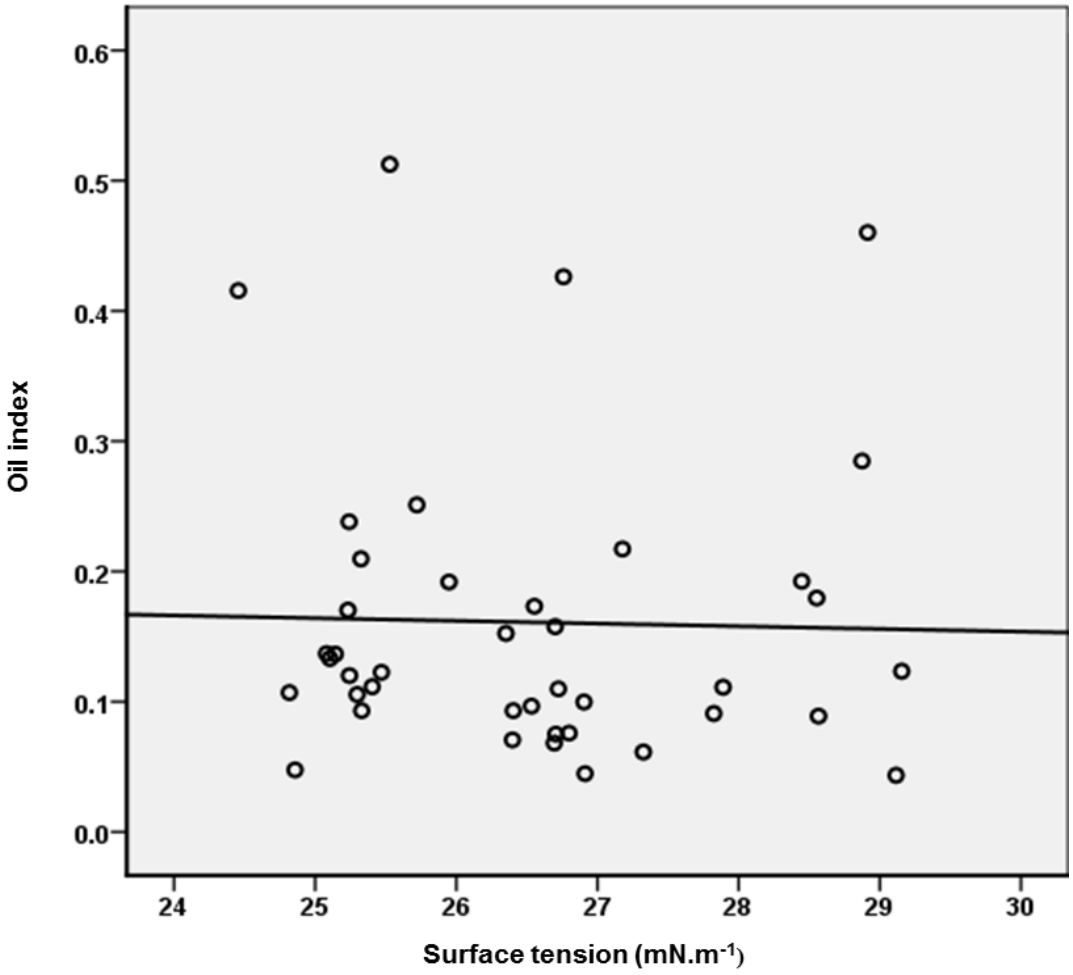

Figure A3.7 Scatter plots of correlations between mean oil index and surface tension of isolates. Shown is the scatter plot of the mean oil index and surface tension of isolates with non-significant interaction amongst growth in the two systems (*P* = 0.879) with a weak correlations (r = 0.429).

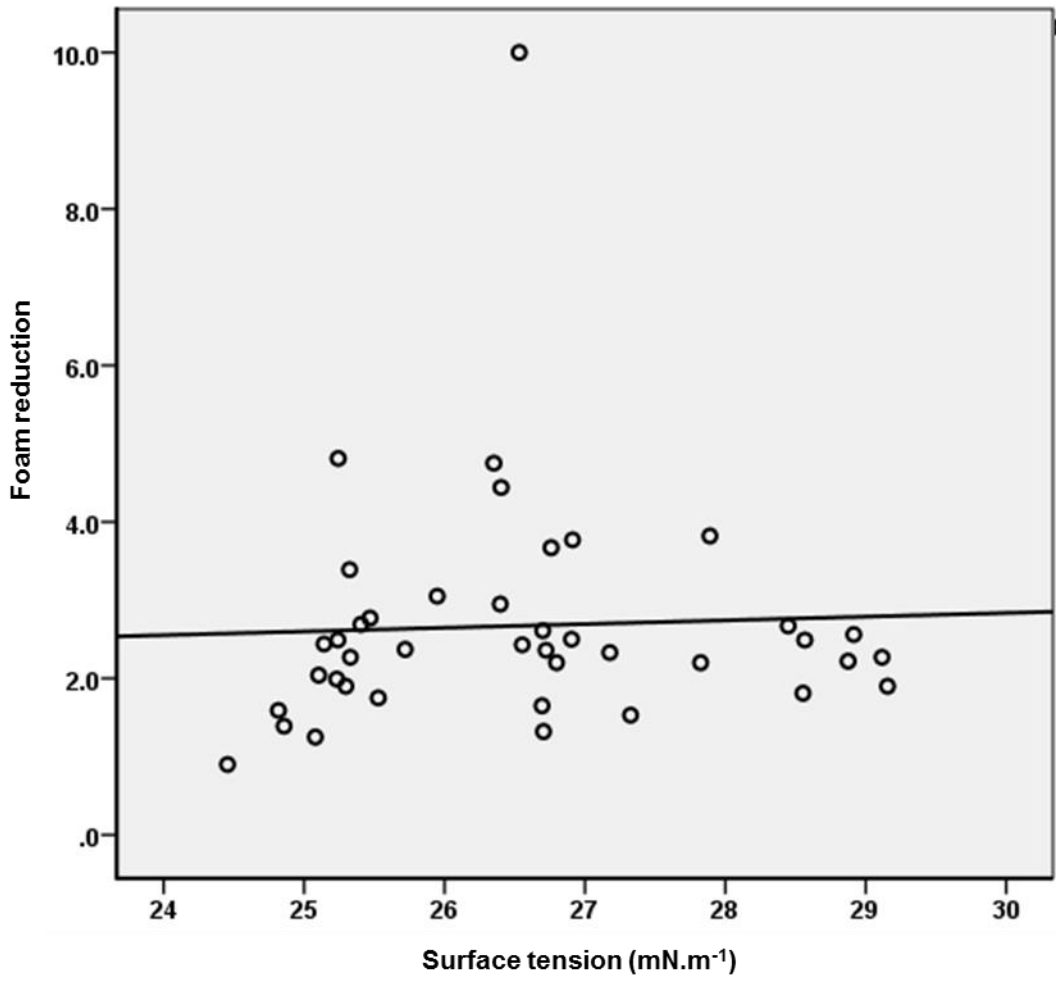

Figure A3.8 Foam reduction and mean surface tension of isolates. Shown is the scatter plot of the foam reduction and mean surface tension with a non-significant interaction between the two systems (*P* = 0.790) and a very weak linear correlations (r = 0.045).

Appendix A4

Additional information for chapter 6

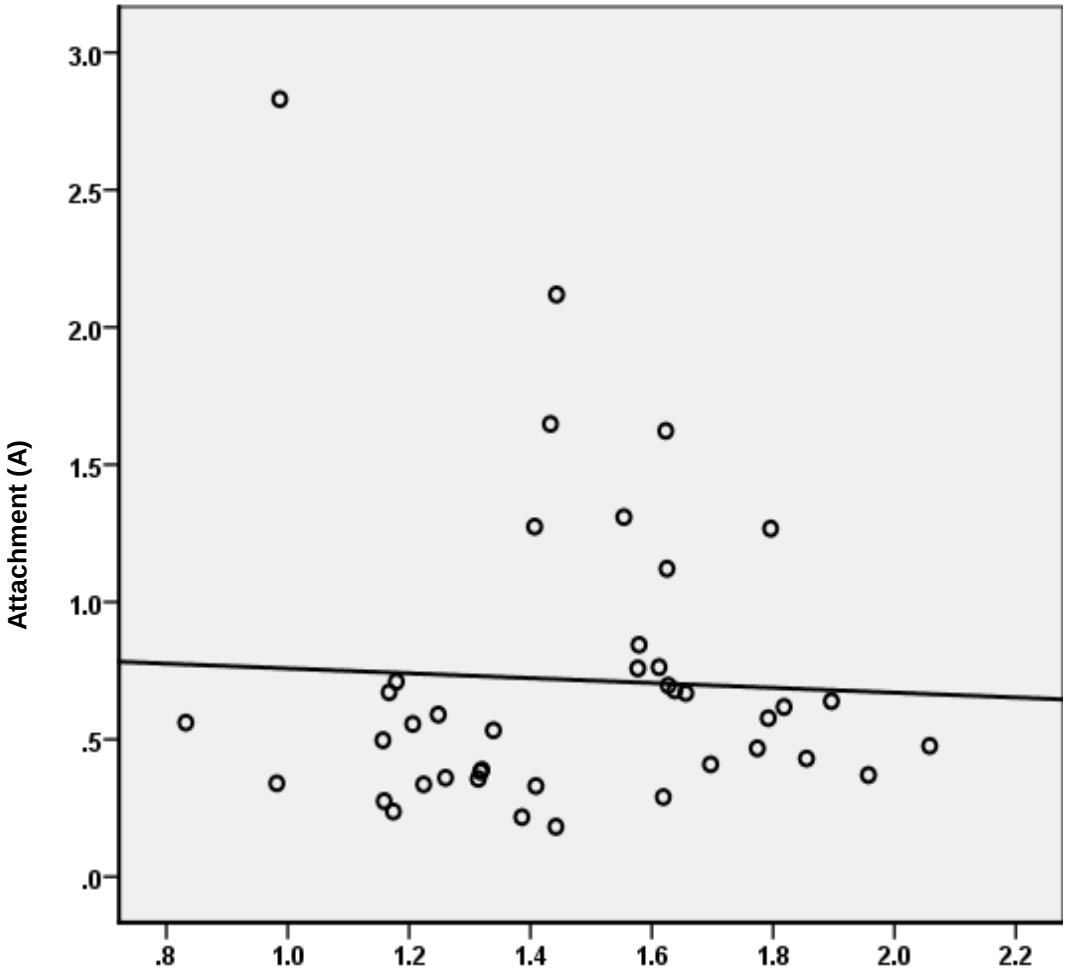

Figure A4.1 Scatter plots of correlations between mean oil index and surface tension of isolates. Shown is the scatter plot of the mean growth of isolates in glass bead column and mean growth in microcosms a non-significant interaction between the two systems (*P* = 0.775) with a very weak linear relationship (r = 0.045).

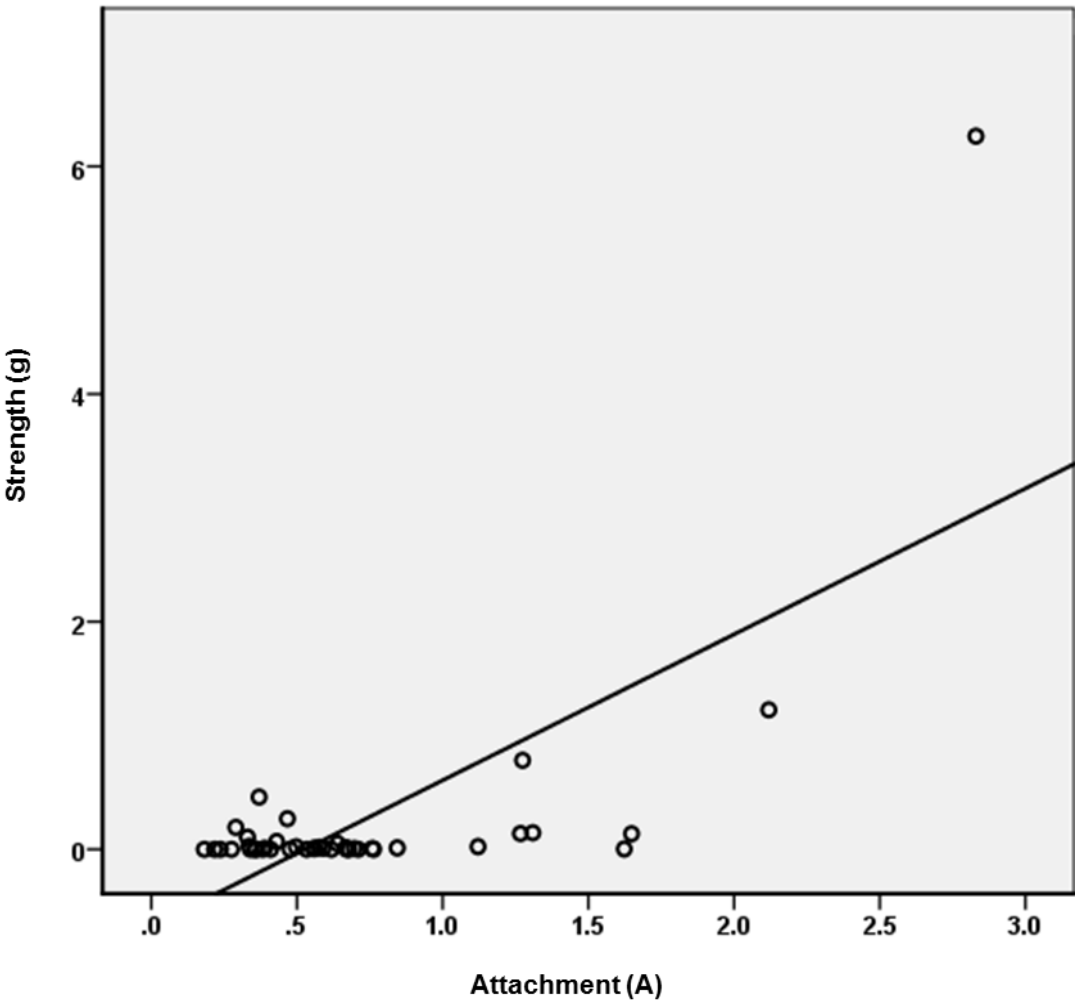

Figure A4.2 Scatter plots of correlations between strength and attachment levels of isolates. Shown is the scatter plot of the mean strength and attachment and a significant interaction between the two systems (*P* = 0.000) with a strong linear relationship (r = 0.706).

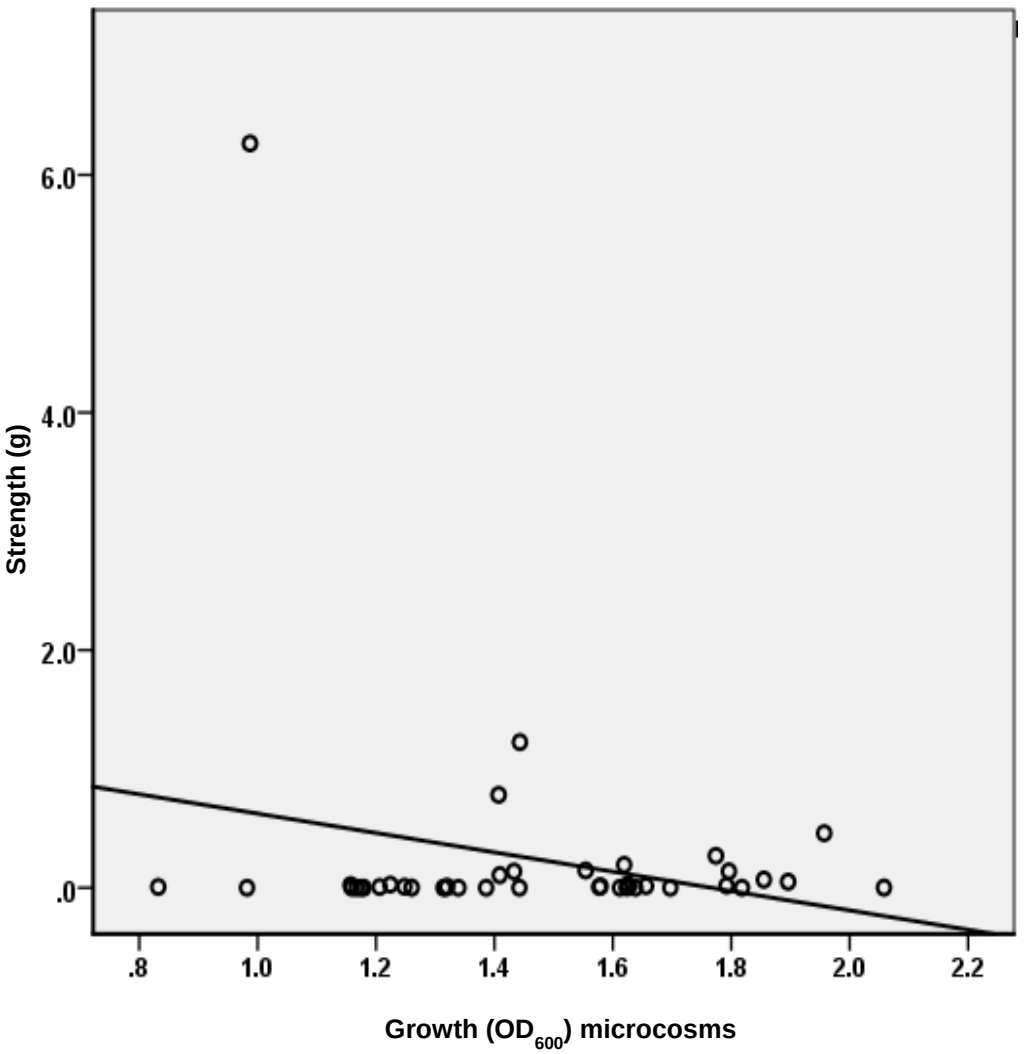

Figure A4.3 Scatter plots of correlations between strength and growth in microcosms. Shown is the scatter plot of the mean strength and growth in microcosms and a non-significant interaction between the two systems (*P* = 0.140) with a very weak linear relationship (r = 0.235).

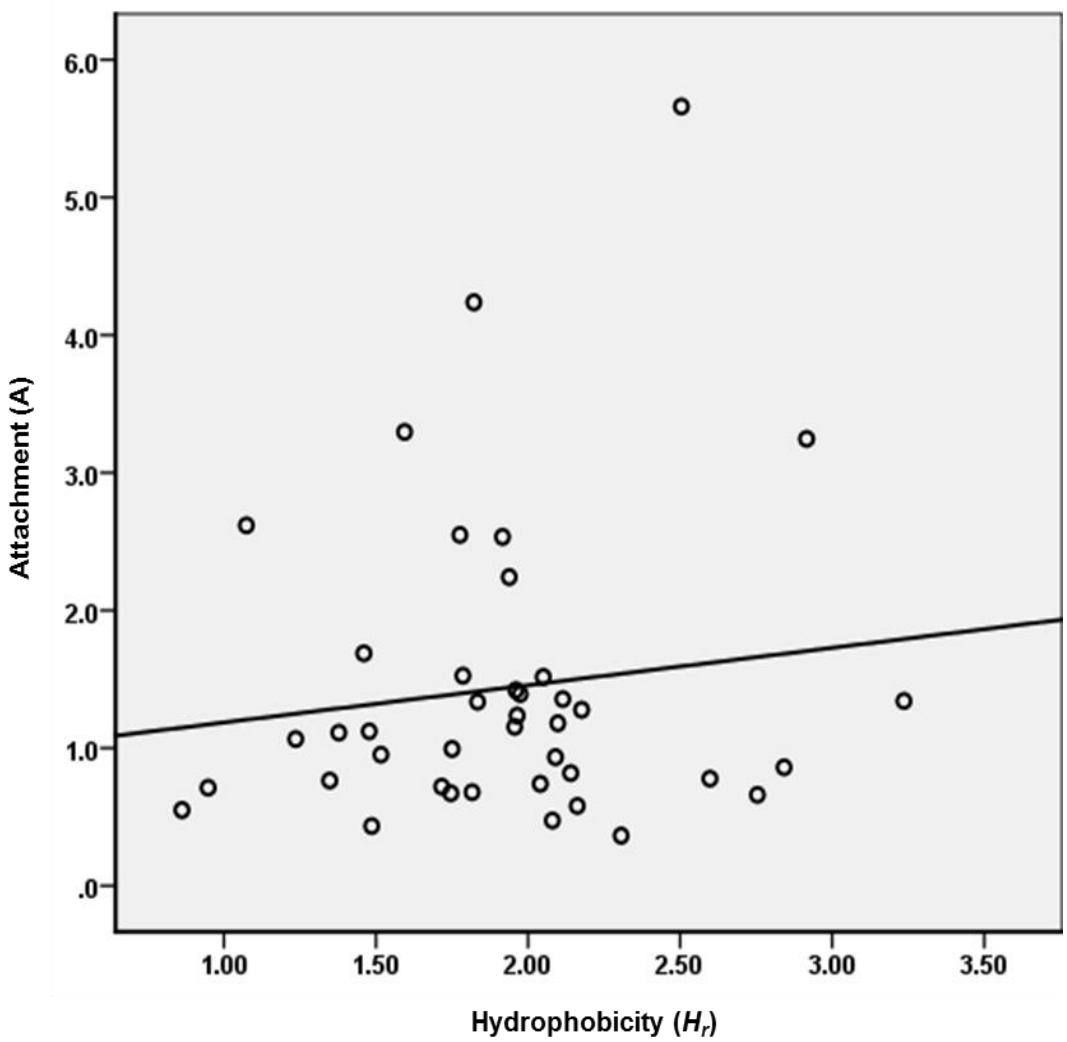

Figure A4.4 Scatter plots of correlations between attachment and hydrophobicity of isolates. Shown is the scatter plot of the mean attachment and hydrophobicity with a non-significant interaction amongst the two systems (*P* = 0.429) and with a very weak linear relationship (r = 0.126).

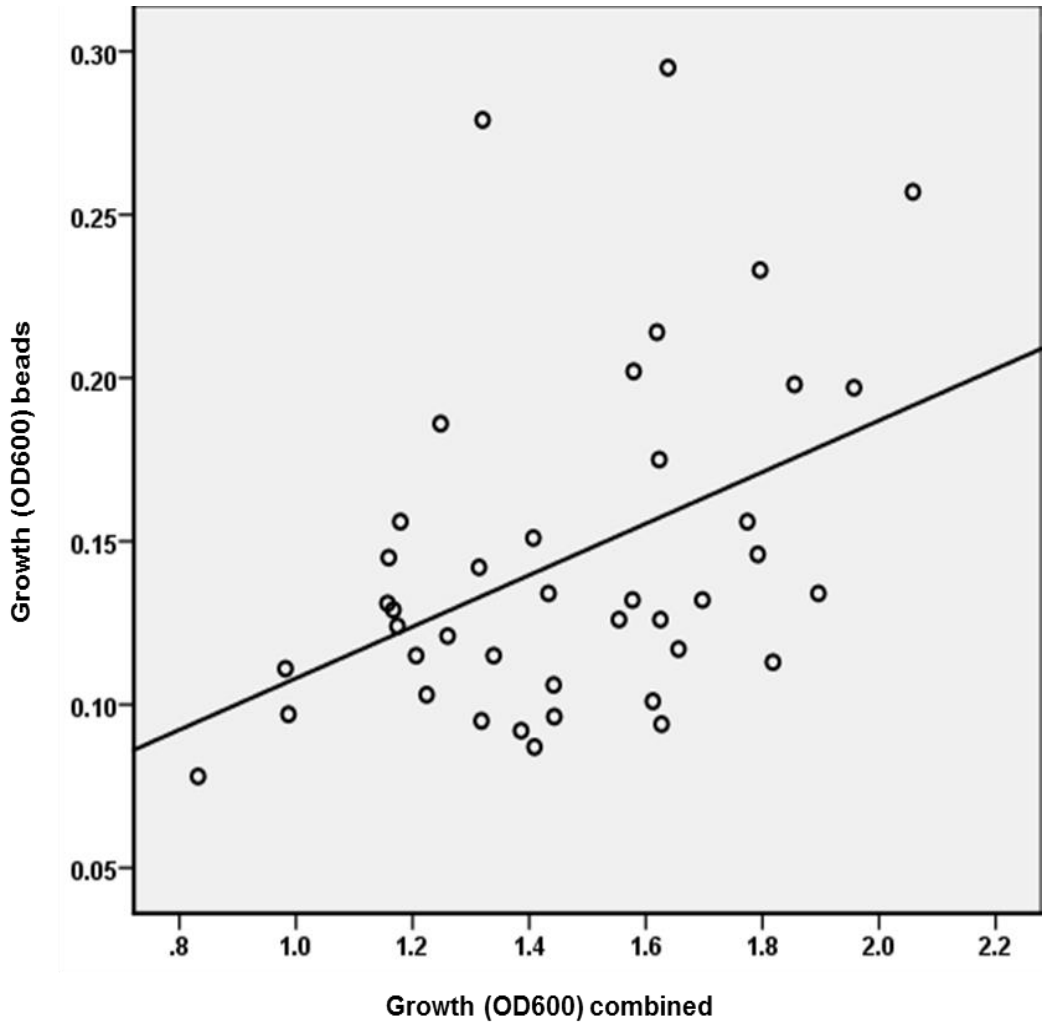

Figure A4.5 Scatter plots of correlations between mean growth in bead column and growth in microcosms of isolates. Shown is the scatter plot of the mean growth of isolates in glass bead column and mean growth in microcosms with a significant interaction between the growth in the two systems (*P* = 0.005) and a weak linear relationship (r = 0.429).

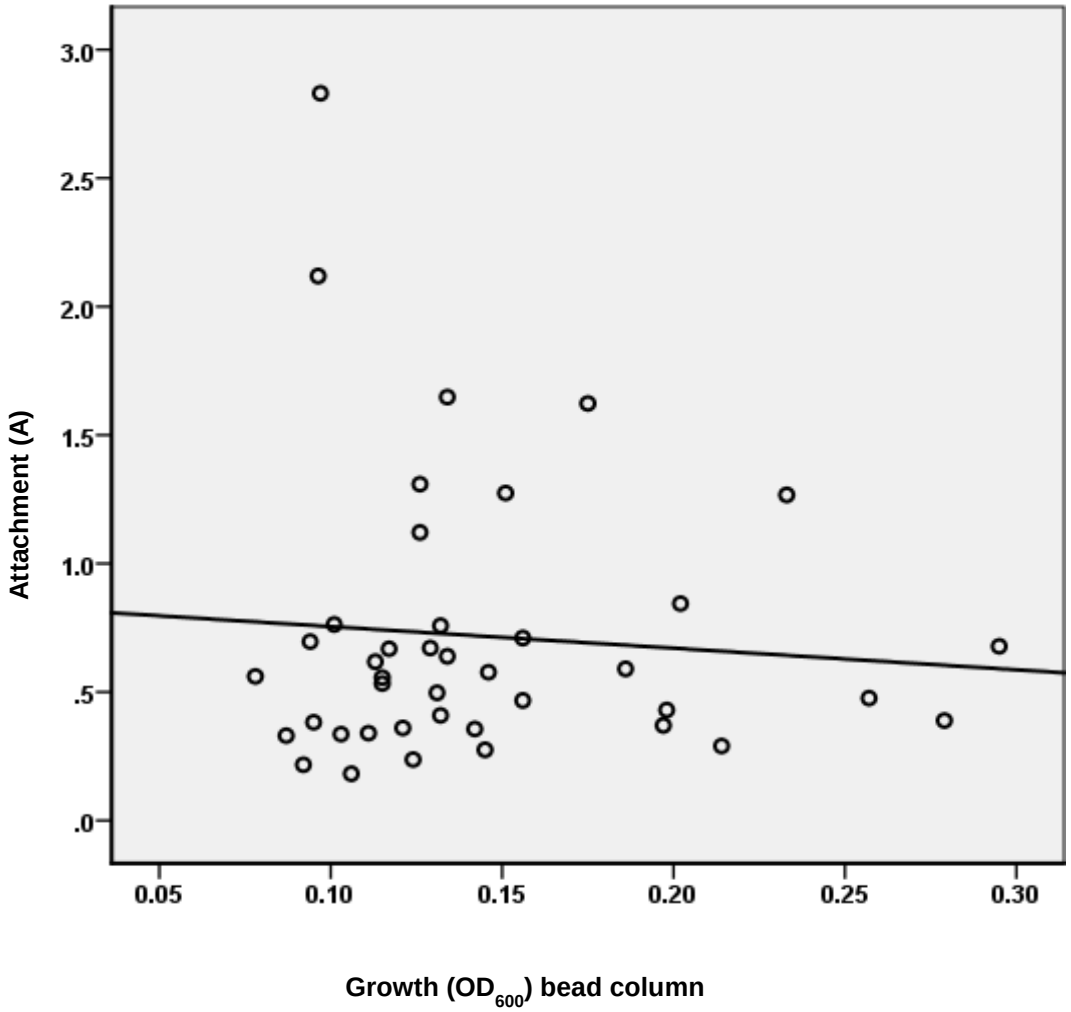

Figure A4.8 Scatter plots of correlations between mean attachment and growth in column bead systems. Shown is the scatter plot of the relationship between mean attachment and mean growth, with a non-significant interactions (*P* = 0.615) and a very weak linear relationship (r = 0.084). This may be as a result of the effects of the outliers.

Appendix A5

Publication related to this work

The experimental work described in this Thesis has been published. A copy is included after this appendix.

Conference:

Ibrahim Mohammed., Yusuf Deeni., Simona Hapca., Andrew Spiers. (2014). ‘’Predicting the minimum liquid surface tension activity of pseudomonads expressing surfactants’’. Poster presentation in Environmental Microbiology Forum. Society for General Microbiology Annual Conference, 14-17th April, 2014.

Published Journal from this work:

Mohammed, I. U., Deeni, Y., Hapca, S. M., McLaughlin, K., Spiers, A. J. (2015). ‘’Predicting the minimum liquid surface tension activity of pseudomonads expressing biosurfactants’’. *Letters in Applied Microbiology* **60**, 37-43.